ECOPHYSIOLOGY OF PATHOGENIC *Pseudomonas spp.* IN SEASONAL AND PERENNIAL PONDS AROUND COCHIN

Dissertation Submitted by

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CONTENTS

CERTIFICATE

Certified that, the dissertation entitled **"Ecophysiology of pathogenic *Psendomonas* spp. in seasonal and perennial ponds around Cochin"** is a bonafide record of work done by **Kum. Pramila S.** under our guidance at the Central Marine Fisheries Research Institute during the tenure of her M.F.Sc. (Mariculture) programme of 1995-97 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.

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DECLARATION

I hereby declare that this thesis entitled "**Ecophysiology of pathogenic *Pseudomonas spp.* in seasonal and perennial ponds around Cochin**" is based on my own research work and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Cochin - 682 014
July, 1997.

Pramila S.

कोचीन के चारों के बहुवर्षी और मौसमी खेतों से नियुक्त रोगजनक स्यूडोमोनास जाति की पारिस्थितिक शरीरक्रिया विज्ञान

वर्ष 1997 के मई - जून महीनों के दौरान किए गए तीन परीक्षणों में बहुवर्षी और पोक्काली खोतों के पानी और तलछट के नमूनों से कुल ।। रोगजनक **स्यूडोमोनास** को वियुक्त किया गया । परीक्षणों से प्राप्ति कुल 13 ओविसडेस पोसिटीव और गलूकोस लेगटीव आइसोलेट्स में तीन हरा प्रतिदीप्ति वर्णक (फलोरसेन्ट पिगमेन्ट) का उत्पादन करने वाले थे । सभी आइसोलेट्स ग्राम नेगटीव छोटे दंड समान थे । ग्लिसरोल और मगनीशियम क्लोराइड की तुलना में डाइपोटासियम हाइड्रजन फोसफेट द्वारा प्रतिदीप्ति वर्णक का उत्पादन बढ़ाया गया । $5^{\circ}C$ तापमान में बढती सामान्य थी और $37^{\circ}C$ तापमान में ।। आइसोलेटों ने अच्छी बढ़ती दिखाई । इन में दो, सिस्टाइन नामक सलफर युक्त अमिनो आसिड से इाइड्रजन सलफाइट का उत्पादन करने की क्षमता वाले थे, परीक्षण किए गए सात नाईट्रोजीनस यौगिकों में केवल अमोणियम क्लोराइड और अमोणियम ओक्सलेट जैसे यौगिकों में अमोणिया तक का निम्नीकरण देखा गया । आस्परजिन और ग्लूटामिक आसिड सबसे कम निम्नीकृत नाइट्रोजीनस यौगिक थे और यूरिया और सिस्टाइन को क्रमशः 60% और 50% आइसोलेटों द्वारा निम्नीकृत किया था कुल 12 आइसोलेटे पेन्सिलिन के प्रयोग में अधिक प्रतिरोधक देखे गए । पाँच संवर्धकों ने परीक्षण रोगजनकों (टेस्ट पाथोजन्स) जैसे **विब्रिया एंग्विल्लेरम, एइवेर्सिसियेल्ला, माइकोबैक्टीरियम और साइटोफेगा** के प्रति संदमन स्वभाव दिखाया ।

PREFACE

Diseases are the most important limiting factors in aquaculture, as in the aquatic medium, all natural barriers that exist between a pathogen and the host are broken down whenever animals are cultured artificially from their natural environment and the animals are really under stress. This makes them more easily susceptible to pathogens and other diseases. The culture ponds are stocked heavily which is another reason for the stress, but the sole purpose of aquaculture is to produce a maximum yield in minimum time as we are in need of more protein. Owing to the widespread loss caused by the pathogens it is important to study the pathogens, the disease caused by them and the methods to eliminate them from aquaculture ponds.

The following 10 families are identified as having members that can produce diseases in cultured animals. These are

a. **Cytophagaceae**

Under this family the Genus *Flexibacter* which causes widespread diseases in fishes starting with formation of white leisions of haemorrhagic necrotic skin ulcers on the fins, gills & tail and then spreading to the other parts, is included.

b. **Myxococcacecae**

Some unidentified bacteria from this family are known to cause diseases in fishes cultured in China.

c. **Pseudomonadaceae**

These are Gram negative rods that produce a wide variety of diseases with a wide host range. The most commonly produced disease is Haemorrhagic Septicaemia, caused by the genus *Pseudomonas*.

d. **Vibrionaceae**

This includes the genus *Vibrio* and *Aeromonas*. produces *Vibriosis* and has a wide host range. Furunculosis in salmons is due to *Aeromonas*.

e. **Actinomycetaceae**

These are acid fast Gram negative rods that include the genus *Mycobacterium* that produces the dreaded Tubercular leisions in fishes.

f. **Enterobacteriaceae**

These produce a wide variety of diseases in fishes including haemorrhagic Septicemia with dermal leisions which may ulcerate, the causative organisms being *Edwardsiella tarda*.

g. **Corynebacteriaceae**

These are Gram-positive forms known as *Renibacterium* causing Granulomatous leisions in Kidney and other tissues.

h. **Streptococcaceae**

Occur in Freshwater and Marine environments, causing haemorrhagic septicaemia in fishes.

i. **Bacillaceae**

These are Gram-positive small rods causing intoxication; occur in freshwater and marine environment.

j. **Neisseriaceae**

This genus, by itself is not considered as a very dangerous pathogen; but is capable of combining with other pathogens to magnify the effects of diseases.

The various diseases seen in fishes are

Furunculosis - caused by *Aeromonas salmonicida* in trouts and salmons in Europe and U.S.A. causes death in the young and juvenile forms and causes furunculosis to develop in muscles of adult fishes.

Tuberculosis - caused by *Mycobacterium* in a wide range of fishes. Its geographic distribution is world-wide.

Symptoms produced include haemorrhages in different parts of body and Anaemia.

Vibriosis - caused by members of Genus *Vibrio*. Has a wide host-range. Includes the species *Vibrio anguillarum* which produces Vibriosis in cells.

Haemorrhagic Septicemia - Caused by members of the genus *Pseudomonas* and also of family enterobacteriaceae. Symptoms include haemorrhage followed by death of blood cells causing anaemia.

Myxobacteriosis - Produced by members of the genus *Myxobacterium*. Widespread in fishes cultured in China.

Flexibacter columnaris also produces diseases in fishes which gives rise to white leisions on the body. Leisions are formed in Fins & Gills first and then spread to the rest of the body.

Many other pathogenic bacteria have also been found, of which only a small group infect culture systems.

Bacterial diseases in crustaceans

Most of the bacterial diseases in crustacea are of secondary etiology but one or two good examples of primary infections are:

a. **Graffkemia disease** produced in lobsters of genus Homarus The pathogen is the bacteria *Acrococcus viridans*

b. **Brown - spot disease** This is a disease that is caused by members of *Pseudomonadaceae* and *Vibrionaceae*. It infects the exoskeleton which is damaged paving way for secondary infection and death ensues. Difficulty in moulting also occur. If no secondary infection sets in, condition eliminates itself at moulting.

Other diseases include - 'Shell rot' in shrimps, Blue-spot disease in crabs etc.

Bacterial diseases in molluscs

Diseases known so far are caused in Bivalves. Bacillary necrosis is caused by members of *Pseudomonadaceae* and *Vibrionaceae* in the larvae of oysters. Reduced motility, settling at bottom etc. are symptoms. Widespread death is caused. In focal necrosis caused by unidentified bacteria in oysters, gaping of shell results.

Infections may be caused by contact or by toxins produced by bacteria when hosts are under stress so that good growth rate and survival will be affected in mariculture operation.

In the present study, ecophysiology and pathogenicity of *Pseudomonas* were studied as members of the

genus *Pseudomonas* are very common in nature and can be isolated easily. They are the causative agents for Haemorrhagic septicaemia in fishes, Brown-spot disease in crustaceans, *Bivalves Bacillary nerosis* in molluscs etc. They are known for their nutritional versatility towards organic compounds of low molecular weight in media totally devoid of organic growth factors. This capacity, combined with a fast growth rate and antagonistic activity against other microbes allow them to predominate in natural media with neutral pH and some organic matter in solution.

I sincerely wish to thank **Dr. V.Chandrika**, Senior Scientist, CMFRI, Kochi for her guidance and timely help throughout the course of the present study, without which the work would not have been possible. I also acknowledge the help and advice provided by the advisory committee members, **Dr. Mary K. Manissery** and **Dr. K. Rengarajan** during the dissertation period. I would like to express my thanks also to **Dr.M. Devaraj**, Director, CMFRI for providing me with all the facilities during the period of work. I also acknowledge with thanks all the help provided by **Dr. C.Susheelan**, officer in charge, PGPM, CMFRI during the entire course of dissertation work.

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INTRODUCTION

Many groups of aerobic heterotrophic bacteria are opportunistic pathogens, leading to heavy mortality in wild and cultured fish and shell fish. It has been shown by several workers that the normal bacterial flora of the fish is a direct reflection of the bacterial population of the environment they inhabit (Horsely, 1973; Sakata *et al.*, 1980). Most of the causative micro organisms are naturally occurring saprophytes and are essentially opportunist pathogens, which invade the leision of a fish host, rendering it susceptible to infection by stress factors or other disease initiating processes.

The external surfaces of aquatic organisms are always in contact with micro-organisms present in the water. Austin and Austin(1987) summarised the bacterial flora of fresh water and marine farms, and observed that as many as 37 bacterial flora were encountered in the former and 40 in the latter ecosystems. Literature concerning the bacterial flora of penaeid prawns appear to be limited. Llobrera and Gacutan (1977) have studied the bacterial flora of *Penaeus monodon* larval culture water. Vanderzant *et al.*, (1977) studied the microbial flora of pond reared brown shrimp (*Penacus aztecus*) in relation to the environment. Bacterial flora of the digestive tract of penaeid prawns such as *P.*

setiferus and *P. japonicus* was studied by Hood *et al.*, (1971) and Yasuda and Kitao (1980) respectively.

Pseudomonas species are frequently associated with fish (Cahill, 1990) and are found on eggs (Bell *et al.*, 1971, Sugita *et al.*, 1988) the skin and gills (Colwell 1962; Horsely 1973) and the intestine (Trust and Sparrow) 1974; Austin and Al Zahrani 1988). Generally, the bacterial flora of fish, including *Pseudomonas, Cytophaga, Flavobacterium, Micrococcus,* and *Acinetobacter* species and others reflect the microbial population of the aquatic habitat and is influenced by factors such as bacterial load in water and salinity. As *Pseudomonas* species are so widespread and numerous they may become involved in disease processes and act as secondary invaders of fish compromised by pathogens or other factors. *Pseudomonas* consist of approximately 27 recognised species among which some have been reported as primary pathogens, principally *P. anguillisceptica* and *P. fluorescens* and there has been one report of *P. chloraphis*, an organism very similar to *P. fluorescens*, causing heavy mortalities among farmed Amago trout *Oncorhynchus rodurus* in Japan (Hatai *et al.*, 1975).

Species of *Pseudomonas, Vibrio, Flavobacterium, Achromobacter* and *Bacillus* predominate in the sea in the order named (Zobell, 1946). This was found to be the case even in tropical culture fields, where also the *Pseudomonas* is a dominant group among the microbial flora (Sahul

Hameed, 1989). Species of *Pseudomonas* which oxidise various kinds of petroleum hydrocarbons has been demonstrated in marine bottom sediments by Zobell *et al.*, (1943). According to the author, *Pseudomonas* which attack aliphatic, aromatic, naphthenic and olefinic hydrocarbons in the presence of free oxygen are widely distributed in the sea water and marine mud. The formation of slimy or viscous growth, which is a common property of bacterium found on the integument of marine fish is primarily pronounced with *Pseudomonas fluorescens* (Sanborn, 1932; Stewart 1932). This was also found to be abundant in slime, mucus and muscle tissue of marine fish [Gibbons (1934); Kiser and Beckwith 1944)].

Precise identification of the organism by taxonomic systems presents certain difficulties (Cowan, 1955); this is nowhere more apparent than, when with the bacterial pathogens in aquaculture, since they are individual members of the discrete groups, and pathogenicity is often the only feature they share. Representative of 25 bacterial genera have been implicated as pathogens of fresh water and marine fish (Austin and Allen Austin, 1985). However, as methods of identification of bacteria improve and classification systems are updated, so the number of genera and species within each genus, which are designated as pathogens constantly change (Frerichs, and Roberts, 1991).

Early results by Shewan *et al.*, (1954) suggested broad differentiation of groups corresponding roughly to genera *Pseudomonas*, *Achromobacter*, *Vibrio* and the family Bacteriaecceae of Bergey'smanual of Determinative Bacteriology (Breed, Murray and Smith, 1957). In the 8th edition of Bergey's manual of determinative bacteriology detailed description of 25 species of *Pseudomonas* are presented and the names of 236 other organisms assigned to the genus, but not as extensively characterised, are given in appendices. An index based on some distinctive observations supplements the taxonomic treatment. It is a difficult task to present a complete list of species for a genera that has suffered considerable nomenclatural hypertrophy, and very likely the grand total of species names is substantially larger than 265 (Palleroni, 1977). A list of properties that have been found useful for internal subdivisions of the genus is given by the above author.

A determination scheme for the identification of certain genera of Gram negative bacteria, with special reference to the Pseudomonadaceae was given by Shewan *et al.*, (1960). The tests used to identify the genus were colony appearance, Gram's staining, morphology, oxidase tests (Kovacs 1956) Antibiotic and 0/129 sensitivity (Shewan *et al.*, 1954), motility, diffusible fluorescent pigment production by Flavobacterium species and by flagella stain (Caseres - Gil).

The procedures for screening of cultures of marine bacteria by Simidu and Aiso (1962) is of use in identification of marine bacterial genera. The major characters of taxonomic importance included in the scheme are Gram's staining, sensitivity to penicillin, pigmentation, fermentative properties in Hugh and Leifsons Glucose medium etc.

The appropriate methodology for the study of these characters have been thoroughly discussed by Stanier *et al.*, (1960) and by Palleroni and Doudroff (1972). The tests of importance in identification include the Cytochrome oxidase test, (which indicate the correlation between the reaction and the presence of Cytochrome in the cells), the sensitivity tests by Shewan *et al.*, and those by Simidu and Aiso (1962), and the inoculation of the tubes with the medium of Hugh and Leifson (1953); one of which was incubated anaerobically under paraffin indicated the type of metabolism. By means of the H & L medium the organisms can be differentiated into four groups. They were further differentiated based on the presence or absence of fluorescent pigment under U.V. light. The production of diffusible pigment can be determined using either the medium of King *et al.*, (1954) or that of Paton (1959).

In the early taxonomic treatments of *Pseudomonas*, pigment production was a character of primary importance in

the genera level. Later many non-pigmented species were included in the genus. Still, pigmentation character retains an important place among the diagnostic traits of some species (Palleroni, 1977).

According to Sneath (1957) the most logical way of classifying bacteria is to compare the overall similarities of the organisms applying the Adansonian principle of giving equal weight to each character. The studies by Floodgate of Torry research station, Aberdeen, showed that, though there are some overlappings, three groups of *Pseudomonas* can be differentiated and these correspond generally to the organisms of group I, II and III and IV as was obtained from the scheme by Shewan *et al.*, (1960).

Another method to identify the genera biochemically is the nitrate reduction and the denitrification characters. Most species of *Pseudomonas* can utilize nitrogenous compounds for growth (Palleroni, 1977; Ostroff and Henry,1939) and it is found that the anaerobic type of metabolism is found to be possible in case denitrification is performed by the genus (Bergey's manual of determinative bacteriology, Eighth edition.)

Proteolytic activity of *Pseudomonas* was noted by Sanborn (1937), which he found to have a direct economic bearing on the keeping quality of fish.

The production of hydrolytic enzymes for substrates like gelatin, starch etc. were also found to be effective in determining the taxonomic status (Palleroni, 1977).According to the same author, growth at different temperature (4^{o}c and 41^{o}c), Arginine hydrolase reaction etc. are also useful.

Sensitivity to antibiotics is another criterion for identification of the genus.(Shewan, Hodgekiss and Liston, 1954). Sensitivity to Penicillin and Novobiocin is to be studied specially.

The chief ways by which bacterial antagonism occur is by alteration of food supply or by reduction of its concentrations or by production of toxic substances, leading to a relationship designated as antibiosis. Waksman (1937) says that, when two organisms are capable of utilizing the same nutrients but are differently affected by environmental conditions, the one organism that finds conditions more suitable, develops more rapidly and thus deprive the other. A strain of *Pseudomonas fluorescens* isolated from water by Lewis (1929) produced in nutrient media a toxin, which inhibited the growth of bacteria, yeasts and moulds. Additional examples of specific and indirect antagonistic effects of microorganisms are given by Waksman (1941).Also an antiviral agent produced by *Pseudomones* Sp. and its activity against fish viruses are given by Kimura *et al.*, (1990). Bacteriocin produced by the Pseudomonads help to

combat, other bacteria present along with them in the system, thus emphasising the beneficial role of *Pseudomonas spp.* in culture systems. (C. Padilla *et al.* 1996)

There is a paucity of information concerning the normal bacterial flora of cultured animals in relation to their environment. In the present study an attempt was made to obtain the normal bacterial flora of perennial and seasonal ponds around Cochin and to identify and isolate the pathogenic *Pseudomonas* species. Also the ecophysiological aspects of the bacteria including the morphology, growth, bio-chemical activities, behaviour in different media, utilization of organic and inorganic compounds for growth, sensitivity to antimicrobial agents and antagonistic activities were studied as information is scanty as far as the *Pseudomonas* of marine origin is concerned.

As the pathogenic bacteriae are to be studied for their occurrence and distribution in order to evolve efficient methods for their control when they behave as opportunist pathogen, the present study was planned to monitor the ecophysiology and pathogenicity of *Pseudomonas spp.* prevalent in perrennial and pokkali field.

PLATE I STATION 1 PERENNIAL POND

PLATE II STATION 2 POKKALI FIELD

MATERIALS AND METHODS

COLLECTION OF WATER AND SEDIMENT SAMPLES

Area of Study:

Water and sediment samples were collected from perennial and seasonal ponds in the Vypeen island located along 9^{o} 55' - 10^{o}10' N and 76^{o} 10' - 76^{o} 20'E (Figure I). The seasonal (prawn cum paddy fields) ponds from which samples were collected is situated near Manjanakkadu near Narakkal. The monsoon crop in such fields is rice and during summer, traditionally the ponds are used for prawn culture. (Plate I) The perennial ponds from where sample collection was done is located in Edavanakkadu. (Plate II)

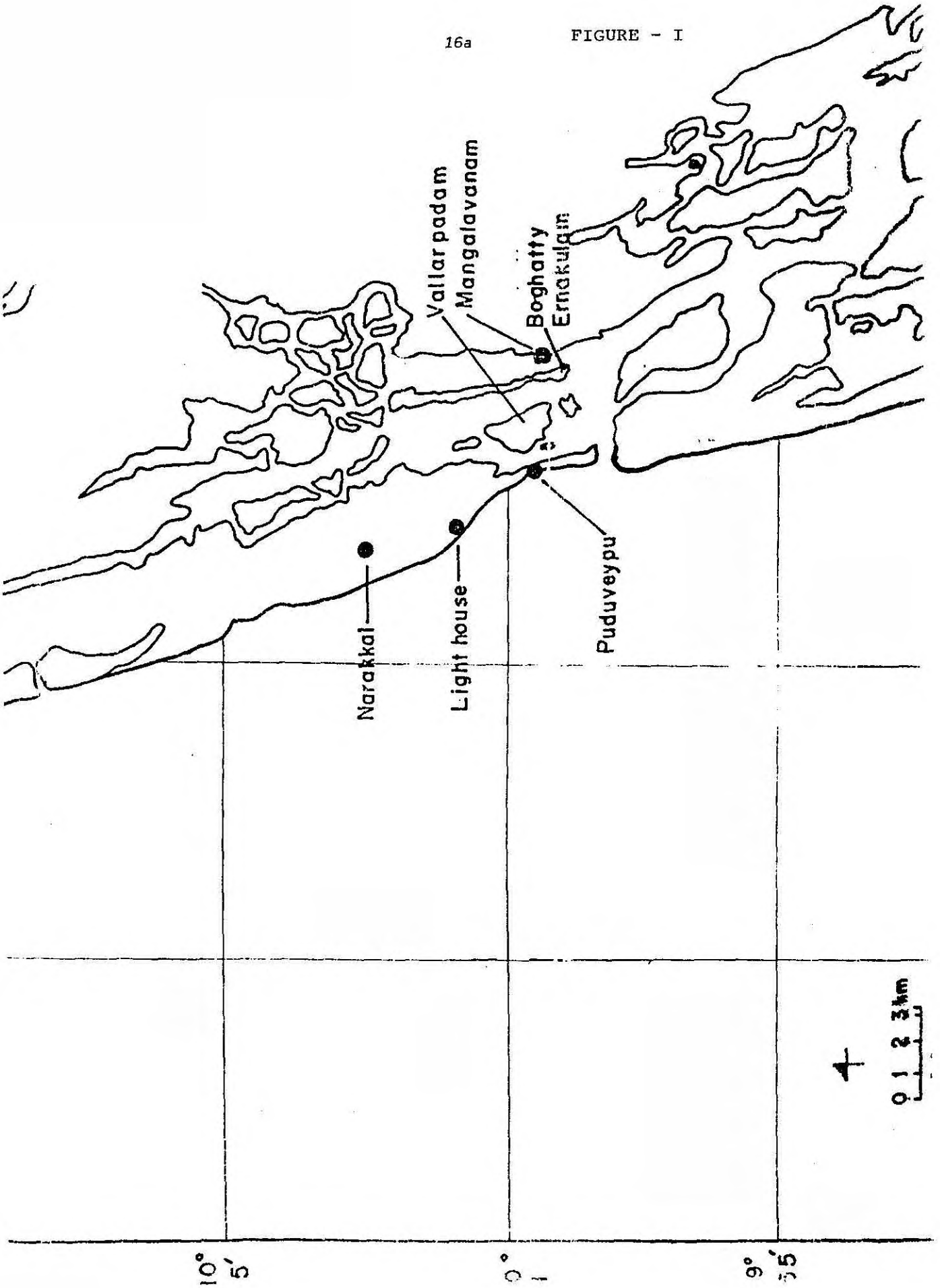
The water samples were collected in screw capped plastic bottles and sediment samples in new polyethene bags. For estimation of dissolved oxygen, fixation was done immediately in 125 ml reagent bottles. Water, temperature and pH were also noted in the field itself. For salinity estimation, the water samples were collected in salinity bottles.

MEASUREMENT OF HYDROLOGICAL PARAMETERS

Temperature

Temperature of water was determined immediately after collection of samples in the fields with an accuracy of $\pm$ 0.1^{o}C using precision mercury thermometer.

FIGURE - I


Vallarpadam
Mangalavanam
Boghatty
Ernakulam
Narakkal
Light house
Puduveypu
0 1 2 3km
10° 5'
9° 55'

Salinity

Water Sample: Argentometry was the method adopted for salinity studies and estimation was done using the formula,

$$\frac{V_2 \times S}{V_1}$$

Where V_2 is the volume of silver nitrate used for titrating 10cc of the sample, S is the salinity of seawater and V_1 is the volume silver nitrate used for titration of standard seawater.

Sediment sample: Sediment and distilled water are mixed in the ratio 1:5 and thoroughly agitated in a shaker for about one hour, after which it was filtered using Whatman's filter paper No.42 and the sediment salinity was estimated by the following formula.

$$\frac{x \times 0.144 \times 35.46 \times 50}{10 \times 10^{*}}$$

Where x is the volume of silver nitrate used for sample, 0.144 the normality of silver nitrate, 50 is, the volume (in millilitres) of distilled water, used for extraction, 10, the volume of extract used for titration, and 10^{*} the amount (in grams) of sediment taken for extraction.

Dissolved Oxygen

Estimation is done by the iodimetric method, using Winkler A (Manganese chloride) and Winkler B (sodium hydroxide and potassium iodide). Samples already fixed using Winkler A and Winkler B are subjected to titration procedure and finally dissolved oxygen values for water samples are obtained from the formula,

$$\frac{V \times N \times 1000 \times 8 \times 1.01}{1.429 \times 1000}$$

Where V is the titre value, N, the normality of sodium thiosulphate, 8, the equivalent weight of oxygen, 1.01 the correction factor, 1.429, weight of 1 ml of oxygen and 100, the volume of sample taken.

pH:

pH of the water samples were determined using the digital pH meter (pH pen).

Isolation of *Pseudomonas* spp.

For estimation of total plate count (TPC) and for isolation of marine Pseudomonads seawater agar (SWA) was used. Selective media like Pseudomonas agar for fluorescein, (Pseudomonas agar, F) and cetrimide agar were also used for initial isolation.

Composition of the above media are as follows:

Seawater Agar

Peptone - 1%; Agar-agar - 2%; Ferric Phosphate - a pinch; Aged seawater - 100 ml; pH 7.2; 15 lbs 30 minutes.

Pseudomonas Agar

Tryptone - 10 g; Peptone - 10 g; dipotassium phosphate - 1.5 g; Magnesium sulphate - 1.5 g; Agar - agar - 15 g; distilled water - 1000 ml. pH 7.2 $\pm$ 0.2; 15 lbs, 15 minutes.

Cetrimide Agar

Beef extract - 10 g; Peptone - 10 g; Sodium chloride - 5 g; cetrimide - 0.3 g; Agar-agar - 12 g; distilled water - 1000 ml. pH at 25^{o}C 7.3 $\pm$ 0.1.

Addition of Triphenyl Tetrazolium Chloride (T.T.C.) to detect Formazan production

Formazan is a water insoluble coloured compound of the general structure, RNH-N=CH^{1}-N=NR^{1}, formed by the reduction of tetrazolium salt in the histochemical demonstration of oxidative enzymes. The R's are usually phenyl groups. In addition, the production of this coloured compound by the addition of TTC into samples demonstrated the fertility of ponds. Isolation was done using media with TTC and those without TTC. The red colour due to formazan

Table I

SERIAL DILUTION TECHNIQUE

Tube No.	1	2	3	4	5
DILUTION	1/10	1/100	1/1,000	1/10,000	1/100,000
Vol. of original fluid (ml)	1	0.1	0.01	0.001	0.0001
Dilution factor	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}

production will increase with increase in cytochrome oxidase positive bacteria.

Estimation of Total Bacterial count -

SERIAL DILUTION TECHNIQUE:

For estimating the total bacterial plate count (TPC), serial dilution technique was used. (Table 1) 99 ml and 9 ml dilutions of seawater were prepared and sterilized, and serial dilutions were prepared for all samples upto 10^5 for water and 10^6 for sediment samples. The inoculation was done by the pour plating method, with the SWA and selective media. The plates were incubated for 24 hours at room temp. (28 $\pm$ 2^{o}C) in a bell jar, in an inverted position. After the incubation period, number of bacteria per millilitre in the case of water sample and number of bacteria per gram in the case of sediment were determined, using the formula,

$$\frac{\text{Count x dilution}}{\text{Weight of sample}}$$

Total plate count (TPC) was taken by counting the number of bacterial colonies in the plates after incubation period of 72 hours.

BIO-DETECTOR MEDIUM

Bio-detector medium TAB-BART^{TM} from Dryocon Bio-concepts Inc. CANADA was inoculated with water samples to detect the fertility of the water.

IDENTIFICATION OF BACTERIA FROM SELECTIVE MEDIA

Morphological features and biochemical and physiological activity were made use of for identifying the bacterial colonies. Morphological characterisation include the examination of size, colour, elevation, margin of the colony, motility, fluorescence, staining properties etc. Biochemical tests basically followed for initial isolation of *Pseudomonas* were the cytochrome oxidase reaction and the type of metabolism of Glucose in Hugh and Leifson's Oxidation Fermentation medium. (Table II & Table III)

For conducting various biochemical and physiological tests, cultures are maintained in seawater agar slants and peptone broth. The cultures are sub-cultured in fresh Agar slants until all biochemical and physiological tests are done.

Seawater Agar Slants

The seawater agar medium was used for maintaining the cultures for testing their physiological and biochemical activity.

Peptone Broth cultures

Peptone water from HIMEDIA having the composition, Peptic digest of animal tissue - 10 g/litre; sodium chloride - 5g/litre (final pH at 25^{o}C, 7.2 $\pm$ 0.2) is prepared and

Table II

PATTERN OF ANALYSIS OF SAMPLES FOR ISOLATION OF *PSEUDOMONAS* Spp.

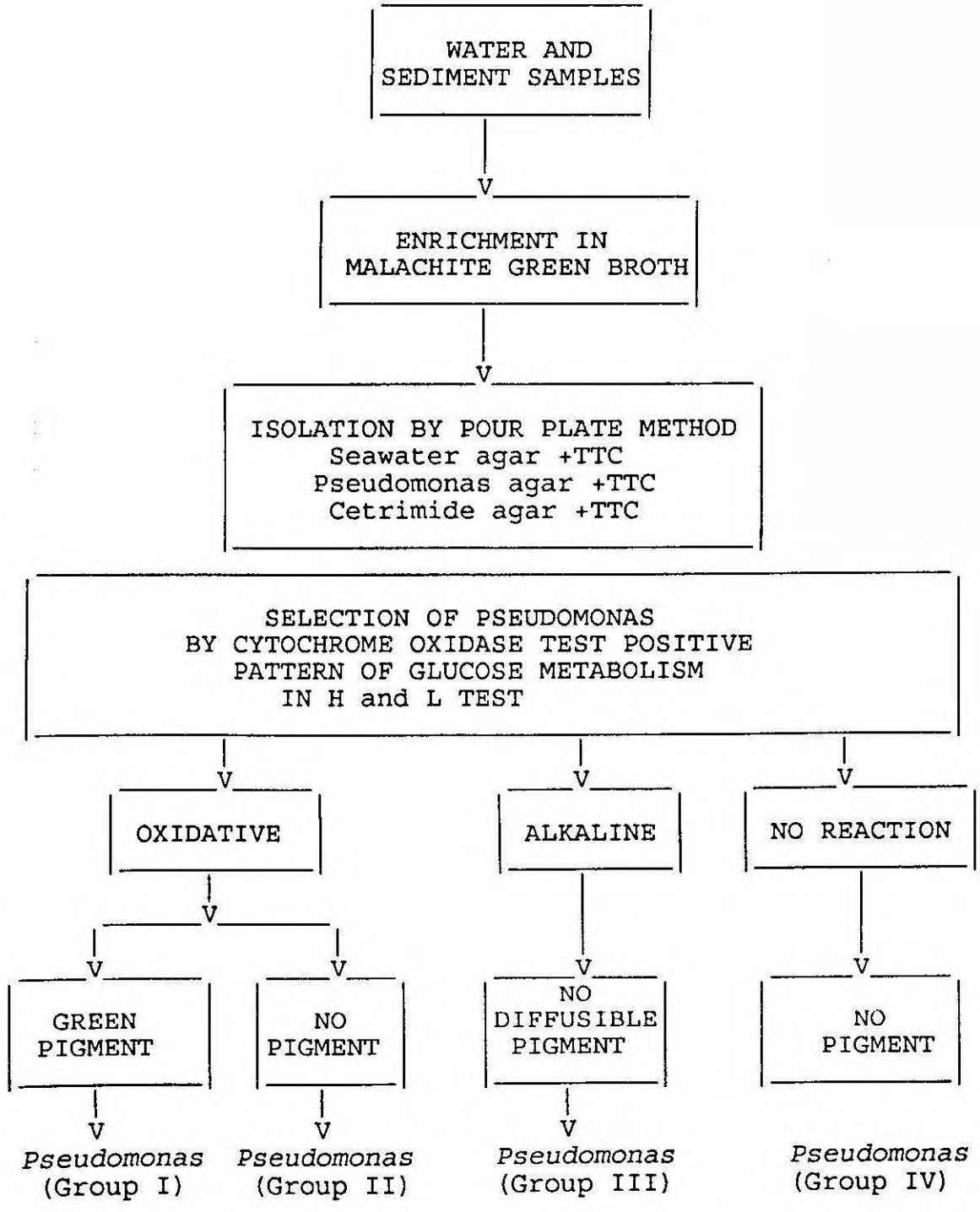
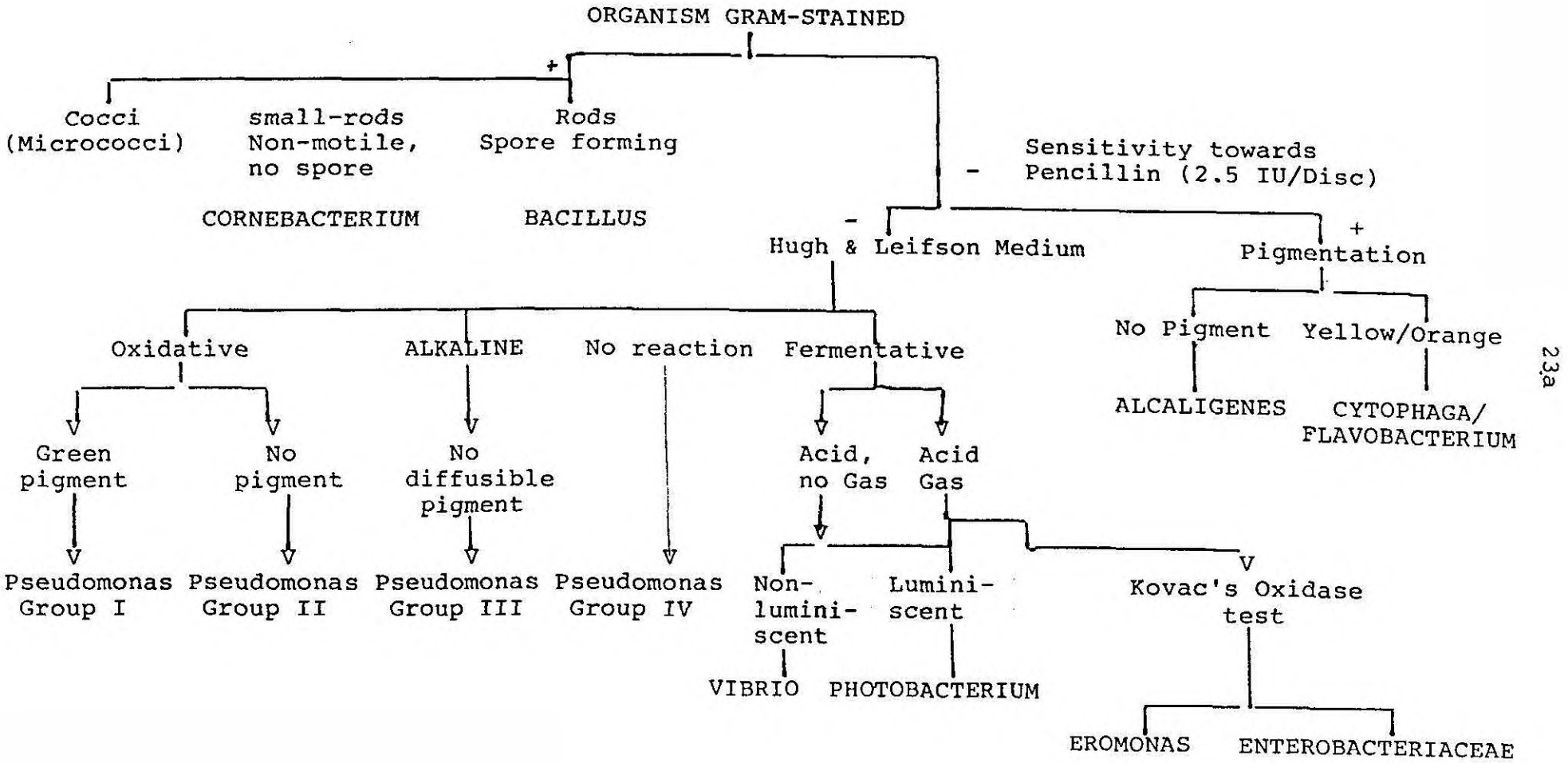


Table III

THE OUTLINE OF PROCEDURE FOR SCREENING OF CULTURES (MARINE BACTERIA) SCHEME OF USIO SIMIDU AND KAYUYOSHI AISO (1962)



inoculated with the 31 isolated cultures for further identification tests.

MORPHOLOGICAL CHARACTERISATION

Size: The approximate size of the colony is measured.

Elevation: The colonies were examined to see if they are elevated or flat.

Colour: The colour of colonies, due to the production of pigments, if any is also noted.

Margin: Margin of the colonies were noticed to find out, if it is uniform or wrinkled. Also the spreading nature, if seen, is also noticed.

Fluorescence: Fluorescence, if exhibited, the colour and nature were studied. Such colonies were viewed under ultra violet light. Fluorescence, is the emission of light by fluorochromes on absorption of an exciting radiation; the wavelength of the emitted light is longer than that of the absorbed radiation and on cessation of irradiation, emission of light ceases within a small fraction of a second. Fluorochromes emit light of characteristic wavelengths. Fluorescence exhibited by *Pseudomonas* spp. was always greenish yellow).

Motility: Motility of the bacteria was checked by hanging drop method.

Hanging Drop Method

Young cultures were used to observe the motility of the bacteria. A little vaseline is smeared around the edge of a clean cover slip and a small loopful of culture is transferred to its centre. A cavity slide is inverted over the cover slip so that the drop of culture will be hanging in the centre of the cavity. Microscopic examination is carried out quickly.

Gram's Stain

Staining was done using the Gram's stains and observed under microscope for cell wall characteristics.

Gram's staining is a differential staining procedure, requiring a primary stain and a counter stain. A thin smear of the culture is prepared and air dried and then heat fixed. The primary staining is with crystal violet followed by the addition of a mordant-iodine. It combines with the primary stain forming a crystal violet iodine complex. In the Gram staining procedure, Gram positive organisms do not retain the primary dye, if iodine is omitted. After decolourising with 95% alcohol, saffranin (Counterstain) is added to the smear. By using this procedure, it is possible to divide the bacteria into Gram positive, Gram-negative, Gram variable and Gram-unreactive.

BIOCHEMICAL TESTS

The oxidase reaction, using the cytochrome oxidase reagent was the primary method tried for classifying the bacterial colonies. Those which were found to be positive in the oxidase test were selected and steaked on agar slants and simultaneously inoculated in peptone water.

CYTOCHROME OXIDASE TEST

1% oxidase reagent, N',N',N' tetra methyl paraphynelene diamine dihydrochloride, which is a redox dye is freshly prepared. For conducting the test, a filter paper strip in which a little reagent is poured is streaked with a loop. Immediate appearance of a deep purple colour indicates positive reaction.

HUGH AND LEIFSON'S OXIDATION FERMENTATION TEST

Oxidase positive bacteria selected in the oxidase test is inoculated in H and L medium, whose composition is as follows: Peptone - 1%, Sodium chloride - 0.5%; glucose - 1%, Agar-agar - 0.3%, distilled water - 100 ml; phenol red- 1CC/100 CC of 0.1% solution; K_2HPO_4 - 0.3%.

Those which were found to be non-fermentative were grouped according to the nature of metabolism as follows: Oxidative type of metabolism is characteristic of two groups of Pseudomonads - *Pseudomonas* group I and *Pseudomonas* Group II. Another group exhibits oxidation with

alkaline end products, which is classified as *Pseudomonas* group III. Those belonging to group IV do not show any change (No reaction) in H & L. medium. They are grouped into Group IV with supporting evidence from additional biochemical characters.

Hydrogen Sulphide Production

L-cystine, the sulphur containing amino acid was prepared in liquid broth alongwith peptone, sodium chloride and beef extract for detection of hydrogen sulphide production by pathogenic *Pseudomonas*. The test organisms were inoculated in the liquid medium and lead acetate paper impregnated with saturated lead acetate solution was retained at the top of the tube. The culture was incubated for 7 days at room temperature and blackening of the lead acetate paper was noted.

The Gram-negative oxidase positive non fermentative rods were classified into respective species by the following tests. (Table IV)

GROWTH AT $5^{o}C$ and $37^{o}C$

Agar slants streaked with the cultures were tested for their growth by incubating them at $37^{o}C$ (in an incubator) and at $5^{o}C$ (in a refrigerator). After the incubation period growth was recorded.

Table IV

SPECIES IDENTIFICATION TESTS FOR PSEUDOMONAS AND OTHER NON-FERMENTATIVE GRAM NEGATIVE RODS

Biochemical identification	Pseudomonas fluorescens	Pseudomonas putida	Pseudomonas anquillisc-eptica	Altermonas piscida	Flavobact-erium spp.	Alcaligenes faecalis
Motility	+	+	+	+	-	+
Diffusible pigment	Green fluorescent	G F	-	-	-	-
Non-diffusible pigment	-	-	-	Yellow	Yellow	-
Growth at 5°C	+	+	+	-	+	+
Growth at 37°C	d	d	-	+	d	+
Growth at 0% Nacl	+	+	-	-	d	+
5% Nacl	+	+	-	+	.	.
7% Nacl	-	-	-	+	.	.
10% Nacl	-	-	-	+	.	-
Sensitivity to Penicillin	R	R	R	R	R	S
Sensitivity to Novobiocin	R	R	S	S	.	R
O-F Medium (glucose)	O	O	-	O	O	Alk
Nitrate reduction	+	+	-	+	+	+
Citrate as C source	+	+	+	-	-	+
Glucose oxidation	+	+	-	+	.	.
Arginine dihydrolase	+	+	-	-	.	-
Hydrolysis of gelatin	+	-	+	+	+	-
Hydrolysis of caesein	+	-	+	+	+	-
Hydrolysis of Starch	-	-	-	+	d	-

All strains are IMViC - - - +
Lysine, Ornithine -
O - Oxidative
Alk- alkaline reaction
R - Resistant
S - Sensitive

. - not known
+ - 90% or more strains positive
- = 90% or more strains negative
d = 11-89% strains positive

GROWTH AT DIFFERENT SALT CONCENTRATIONS

Sodium chloride was added to the peptone broth at concentrations of 5%, 7% and 10% and inoculated to test their salt-tolerance. Another set of cultures were also inoculated in medium without sodium chloride. After the incubation period growth in the media was recorded.

UTILIZATION OF NITROGENOUS COMPOUNDS

A large number of *Pseudomonas* sp. are known to possess the property of denitrification. It includes all the processes, in which nitrate disappears under the influence of bacterial enzyme action and appears in some less oxidised state. Most bacterial species do not carry out the reduction beyond the stage of nitrite. However in some instances, the reduction may proceed to the ammonia stage, and even to molecular nitrogen. Several groups of organisms are involved in the reduction of nitrtate to nitrite to ammonia and finally to molecular nitrogen.

The reduction of nitrate proceeds only in the presence of an anaerobic environment. Nitrate supports anaerobic growth by acting as a hydrogen aceptor. The test is of value in identifying and classifying bacteria.

Utilization of nitrogenous substrates like cystein, ammonium chloride, urea, ammonium oxalate, glutamic acid and asparagine, Aniline etc. were tested in an ordinary

peptone broth containing glucose and inoculated with culture. Turbidity is checked after the incubation period and the nitrate reduction using nitrate reagent (sulphanilic acid and alpha naphthylamine) and ammonia production (using Nessler's reagent) tests were conducted and results were recorded.

HYDROLYSIS OF ORGANIC COMPOUNDS

Starch, Gelatin and caesein media were prepared according to the composition given and tests were conducted.

Starch Medium: Soluble starch - 0.2%, beef extract - 0.3%; Agar agar - 1.5%, seawater-100 ml; pH 7.2, 15 lbs, 30 minutes.

The starch medium is prepared and sterilized and poured into plates. After cooling culture is streaked on the medium and incubated at temperature. To conduct the test, the plates are inverted and a few iodine crystals are placed on the petridish cover and slightly warmed. Clear area in the medium shows the starch hydrolysis.

Gelatin Medium: Peptone 1%; Beef extract - 0.2%; gelatin - 12%; seawater - 100 ml. Medium is sterilised by Tyndallisation by keeping the medium in streaming steam for 3 consecutive days.

Gelatin medium was inoculated heavily with the test organism by stabinoculation and incubated for 72 hours.

After the incubation period the hydrolysis of gelatin is indicated by "liquifaction" of the gelatin, but as normal preparations of gelatin are liquid at temperatures above 25°C, test media incubated at above 25°C were chilled until the control solidifies before observations on liquifaction are made.

CASEIN: Peptic digests of animal tissue - 10 g/litre, sodium chloride 5g/litre, caesein - 1%. Caesein medium in tubes is also inoculated with a loopful of culture and incubated. After the incubation period, nitrate reduction and ammonia production tests were carried out.

UTILISATION OF CITRATE

The citrate utilization is found out by inoculating the cultures on slopes made from Simmon's citrate agar having the composition; Magnesium sulphate 0.2g, Ammonium phosphate - 1g; Dipotassium phosphate - 1 g; Sodium citrate - 2 g; Sodium Chloride - 5 g; Agar-agar - 15 g; Bromothymol blue - 0.08 g; pH 6.8 $\pm$ 0.2.

After the incubation period at 37°C the positive reaction (utilisation of citrate) is shown by the development of the blue colour in the medium.

ARGININE DIHYDROLASE

The medium for finding Arginine utilization was prepared as follows:

Peptone - 10 g/litre; sodium chloride - 5 g/litre; Arginine - 1%. The medium is dispensed into tubes and sterilized. Cultures were inoculated in the tubes and incubated. After the incubation period (96 hours) nitrate reduction test and ammonia production tests were conducted.

ANTIBIOTIC SENSITIVITY TESTS

Since sensitivity studies play an important role in identifying *Pseudomonas* spp., antibiotics sensitivity studies were conducted with young culture to find the resistances of *Pseudomonas* spp. to various antibiotics. In the present study sensitivity to Penicillin (15 mcg /disc), Kanamycin (30 mcg /disc), Ampicillin (30 mcg/disc) etc. were studied. The test cultures were swabbed on the seawater agar media and the discs were put on the surface of the medium and the plates were incubated. The antibiotics diffuses through the agar occupying a circular zone around the original spot. The bacteria grows on the agar surface in all places except in the circular zone where the antibiotic is present. Size of the zone is related to concentration of the antibiotic.

Generally the antibiotic action is felt in the logarithmic period of growth. Hence a relatively young culture was used for the experiments.

Mechanism of resistance in *Pseudomonas* to Penicillin may depend on

1. The formation of extracellular penicillinase by bacteria.
2. The distruction of Penicillin inside the cell which is not necesarily by Penicillinase.
3. Low activity of the Penicillin binding component.

The activity of antibiotics is expressed in international units (IU). Thus for sample, 1 1U of Penicillin (Oxford unit) is the smallest amount of preparation inhibiting the growth of a standard *Staphylococus aureus* strains. Recently, the method of determining the activity of antibiotics according to the weight of the preparation has received wide application.

One unit of activity (AU) corresponds to the activity 0.6 micrograms (mcg) of the chemically pure crystalline sodium salt of benzyl penicillin. Consequently in 1 mg of sodium salt of benzyl penicillin there may be 1667 AV and in 1 mg of potassium salt 1600 AV. For practical purposes both preparations are manufactured with an activity not less than 1550 AV.

ANTAGONISTIC ACTIVITY STUDIES

Cross streaking method and agar diffusion (Well Plate) methods were used to find out the inhibitory effect on some test pathogens, like *Vibrio anguillarum* (H^{10}), (Source: Central Public Health Laboratory, London)

Cytophaga, Edwardsiella and *Mycobacterium* (isolated in the laboratory itself).

The *Pseudomonas* cultures obtained were inocubated in peptone water in conical flasks and shaked thoroughly for two hours in a mechanical shaker. About 10ml of these broth cultures were taken in centrifuge tubes and then centrifuged for 10 minutes at 2000 rpm. The supernatent of the cultures were swabbed on the seawater agar surface to test the antagonistic activity. Wells were cut in the medium with sterile steel rods of 3mm diameter in which culture extract of the test pathogens are poured and incubated. The antagonistic effect, if any, is shown by the absence of growth of the culture in the well (test Pathogen), in the swabbed area. The zone of inhibition (diameter) was measured in millimetres. (excluding diameter of the cup-3mm) after the incubation period (24 hours). Seawater agar was the medium used for the test, as it was found best for isolation,maintenance and also to test antagonistic activity in the present study.

RESULTS

HYDROLOGICAL PARAMETERS

Surface water temperatures were observed and recorded for all the three collections made during May-June, 1997. The lowest temperature was observed in the third collection, when both perennial and Pokkali fields recorded 32.5^{o}C. A decline in temperature was observed during the late June collection. The highest temperature was observed during early June, when 34.8^{o}C and 35^{o}C were recorded from Pokkali and perennial fields respectively. Salinity was found to be almost constant like pH, among the hydrological parameters studied for the first two collections. Salinity ranged from 16.96 ppt to 18.8 ppt in perennial pond surface water. In Pokkali field it ranged from 17.3 ppt to 18.03 ppt, and sediment salinity ranged from 3.57 ppt to 4.8 ppt in perennial pond and 7.65 to 6.12 ppt in Pokkali fields. But in the third sample it went down to 8.5 ppt for perennial ponds and 13.5 ppt for Pokkali fields. For sediment the values were 2.5 ppt and 2.8 ppt respectively.

Dissolved Oxygen (DO) in surface water of perennial pond ranged from 4.92 ml/lit in first observation during early may, but in second collection in Early June, a rapid decline in Dissolved Oxygen was noticed, the value being 0.83 ml/lit. In the third observation a low value of 1.95 ml/Lt. was recorded.

In the Pokkali fields Dissolved Oxygen was recorded as 4.68 ml/lit in the first observation. In the second and third observation, DO levels were significantly reduced, the value being 1.76 ml/litre and 1.44 ml/litre respectively. pH of the water in perennial pond ranged from 6.9 in the third observation to 7.3 in the first observation. Pokkali field surface water pH ranged from 6.0 in third observation to 7.2 in first observation. In the second observation values for both the fields were 6.6.

QUANTITATIVE DISTRIBUTION OF TOTAL BACTERIAL COUNT (TPC)

The method used in the present study for the cultivation of pathogenic *Pseudomonas* are discussed in detail in several papers and manuals. (Zobell, 1946, Rodina, 1972 and MERCK culture media hand book).

Total bacterial count in perennial pond in the surface water ranged from 9 x 10^3/ml in early May to 140 x 10^3/ml in late June in seawater agar medium (figure II) When triphenyl tetrazolium chloride (TTC) was supplemented to SWA medium, the same ranged from 60 x 10^3/ml to 300 x 10^3/ml during late June. The count in Pseudomonas agar was very low in the final observation, ie. in June, the count being 36 x 10^3/ml. The highest count was recorded in Early June which was 106 x 10^3/ml. When Pseudomonas agar was supplemented with TTC, the count was more when compared to ordinary PA, and it ranged from 14 x 10^3/ml (during late May) to 120 x 10^3/ml (during early June).

FIGURE - II

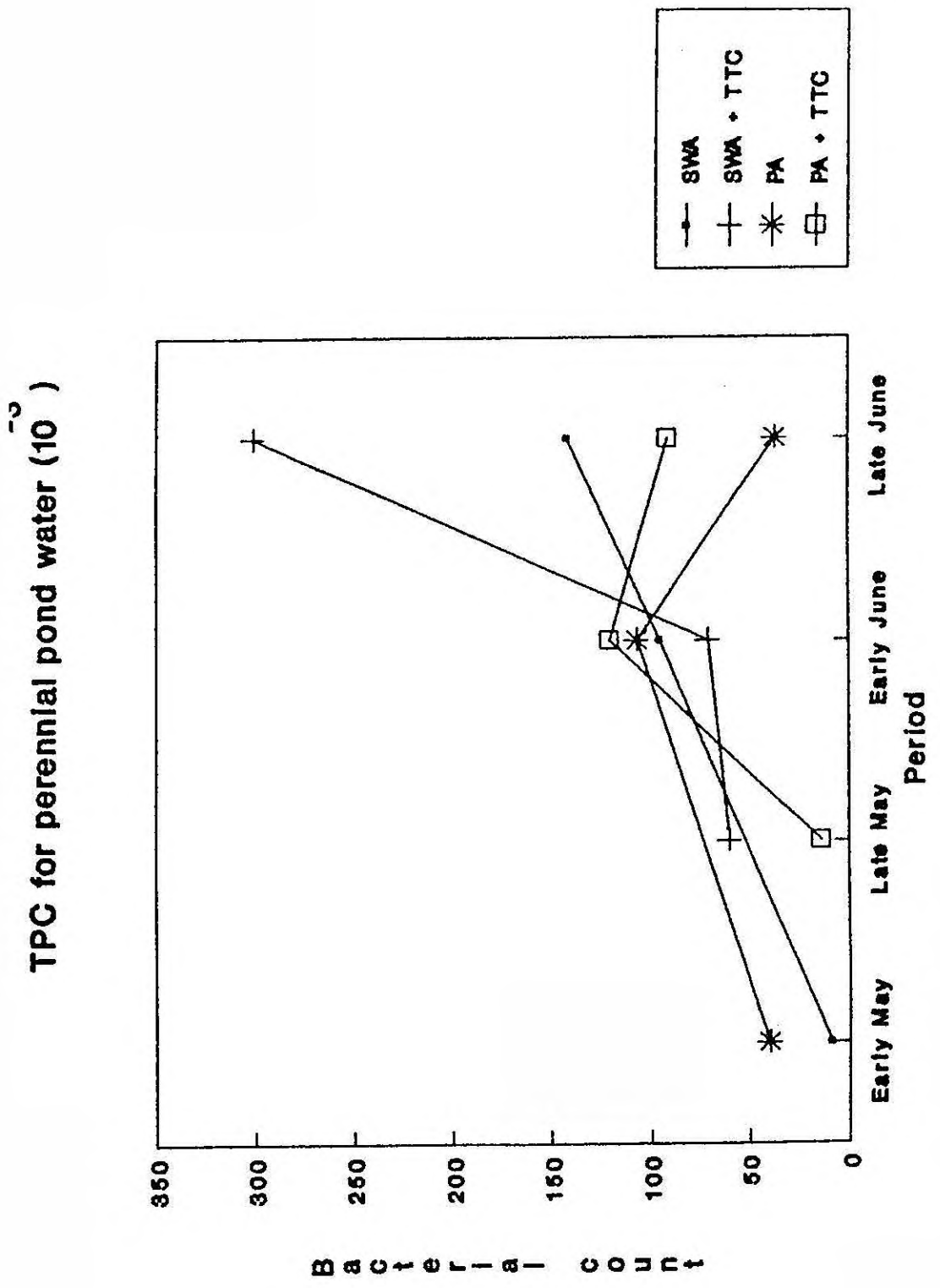

TPC for perennial pond water (10⁻⁵)
SWA
SWA + TTC
PA
PA + TTC
Bacterial count
0
50
100
150
200
250
300
350
Early May
Late May
Early June
Late June
Period

FIGURE - III

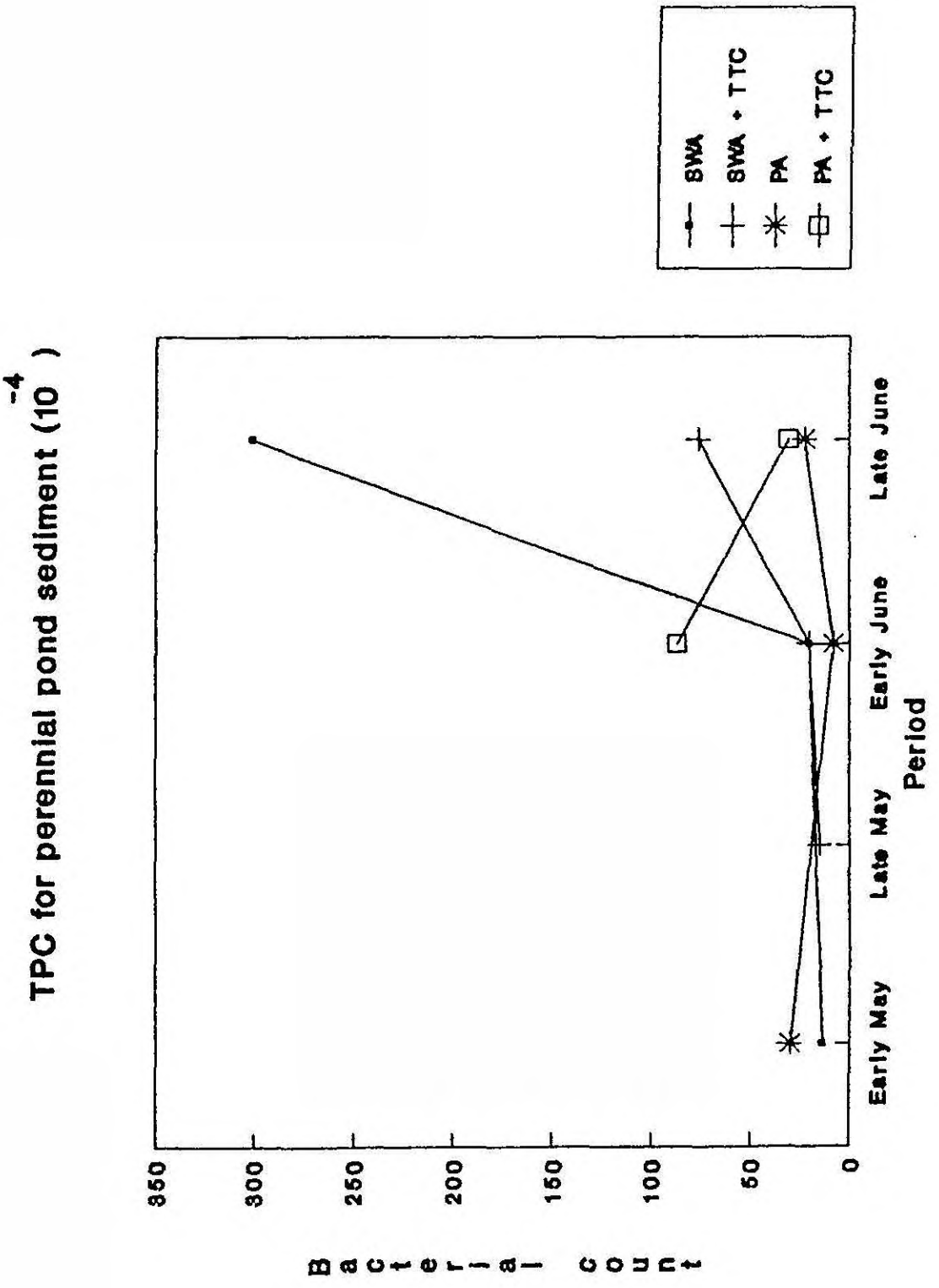

TPC for perennial pond sediment (10 -4)
SWA
SWA + TTC
PA
PA + TTC
Bacterial count
350
300
250
200
150
100
50
0
Early May
Late May
Early June
Late June
Period

FIGURE - III

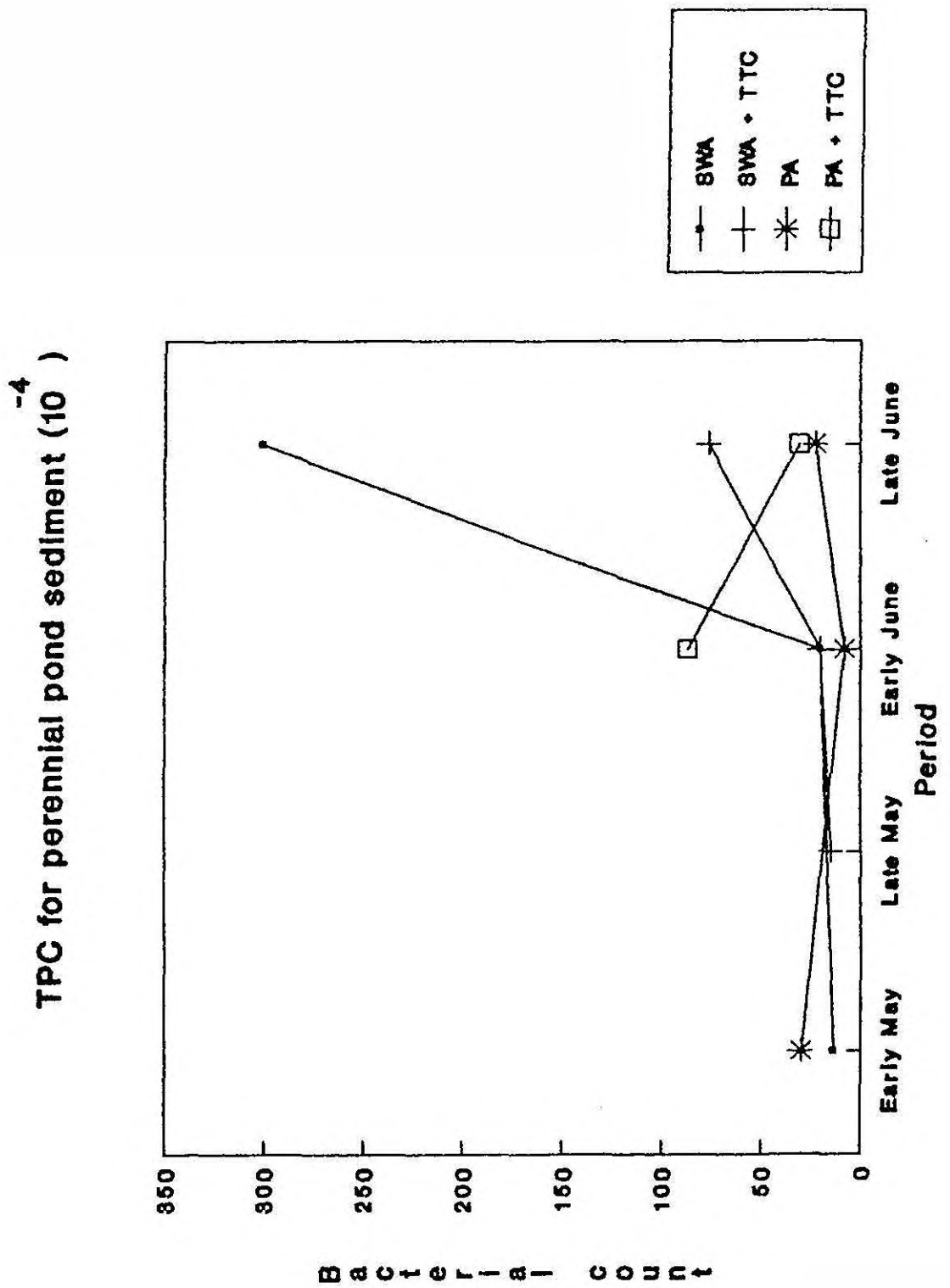

TPC for perennial pond sediment (10^{-4})
SWA
SWA + TTC
PA
PA + TTC
Bacterial count
350
300
250
200
150
100
50
0
Early May
Late May
Early June
Late June
Period

FIGURE - IV

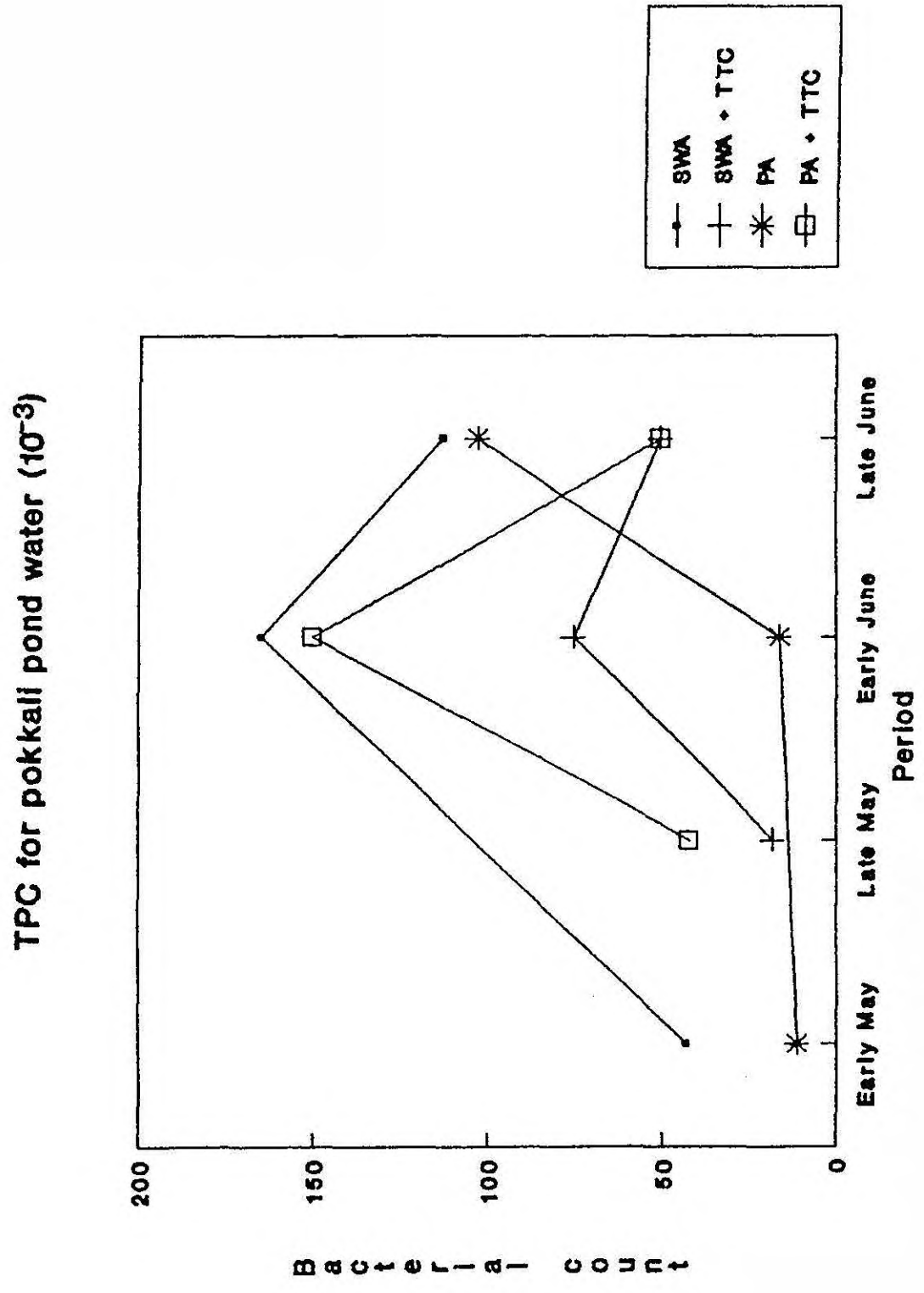
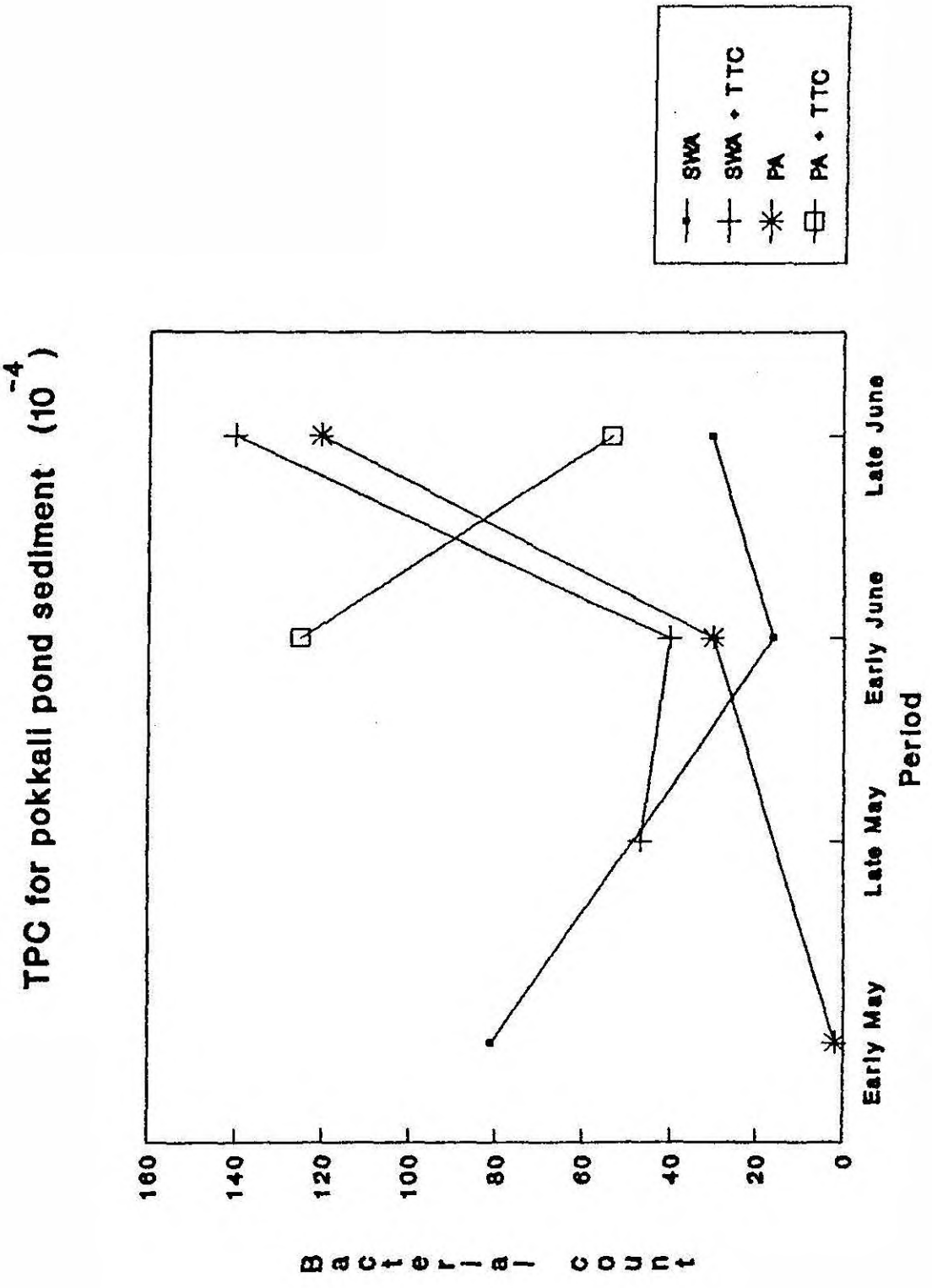

TPC for pokkali pond water (10^{-3})
SWA
SWA + TTC
PA
PA + TTC
200
150
100
50
0
Bacterial count
Early May
Late May
Early June
Late June
Period

FIGURE - V


TPC for pokkali pond sediment (10^-4)
SWA
SWA + TTC
PA
PA + TTC
Bacterial count
0
20
40
60
80
100
120
140
160
Early May
Late May
Early June
Late June
Period

In the perennial pond sediment, the count was very low throughout the observation in seawater agar except in late June, when the count recorded was as high as 300 x 10^4/gram. In the present case, when SWA was supplemented with TTC, the counts recorded were very low except in late June, the count being 75 x 10^4/g. Pseudomonas agar gave very poor results (Figure III).

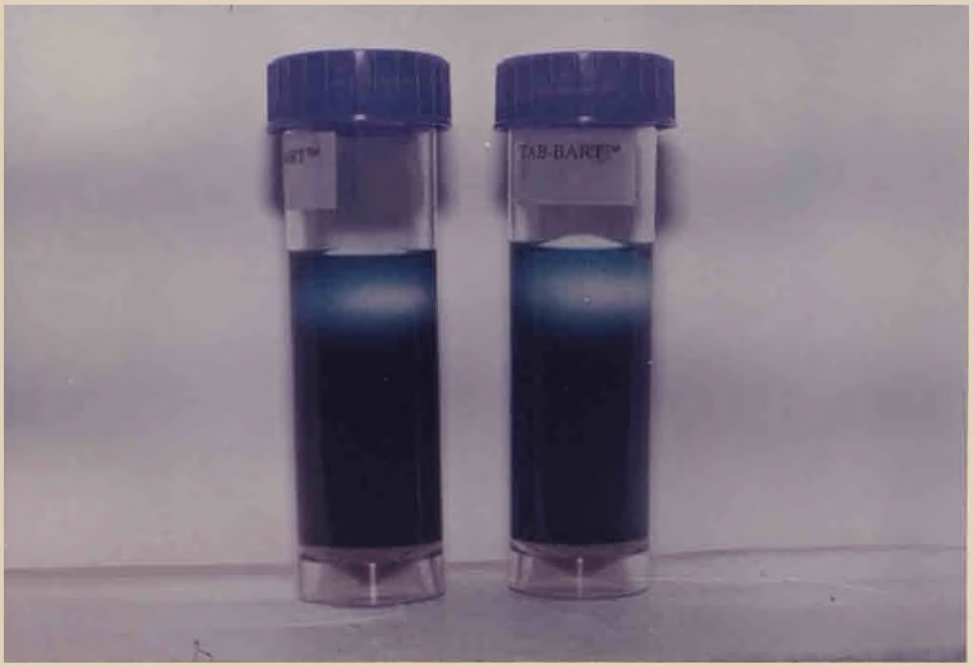
Pokkali field surface water recorded moderate count during June. The counts ranged between 43 x 10^3/ml in early May to 165 x 10^3/ml in early June. TTC supplemented SWA recorded low counts, when compared to non supplemented SWA. PA supplemented with TTC registered high counts in early June the count being 150 x 10^3/ml. Non supplemented PA recorded low counts except in late June, the counts being 102 x 10^3/ml (Figure IV).

In Pokkali sediment, generally count was very low in all the four observations. Seawater agar supplemented with TTC gave the best results; Highest count was obtained in late June in SWA supplemented with TTC, which was 140 x 10^4/g. Regarding PA also highest count was in late June which was 120 x 10^4/g. When supplemented with TTC higher counts upto 125 x 10^4/gm were obtained (Figure V).

Biodetector medium for total aerobic bacteria TAB BART^{TM} from Droycon Bioconcepts Inc. CANADA medium in tubes was inoculated with perennial pond and Pokkali field surface water samples. The reaction was noted after 24 hours. The

PLATE III BIO INDICATOR TAB-BART MEDIUM FROM DRYOCON INC, CANADA

PLATE IV GROWTH AFTER 24 HOURS IN TAB-BART MEDIUM


TAB-BART™

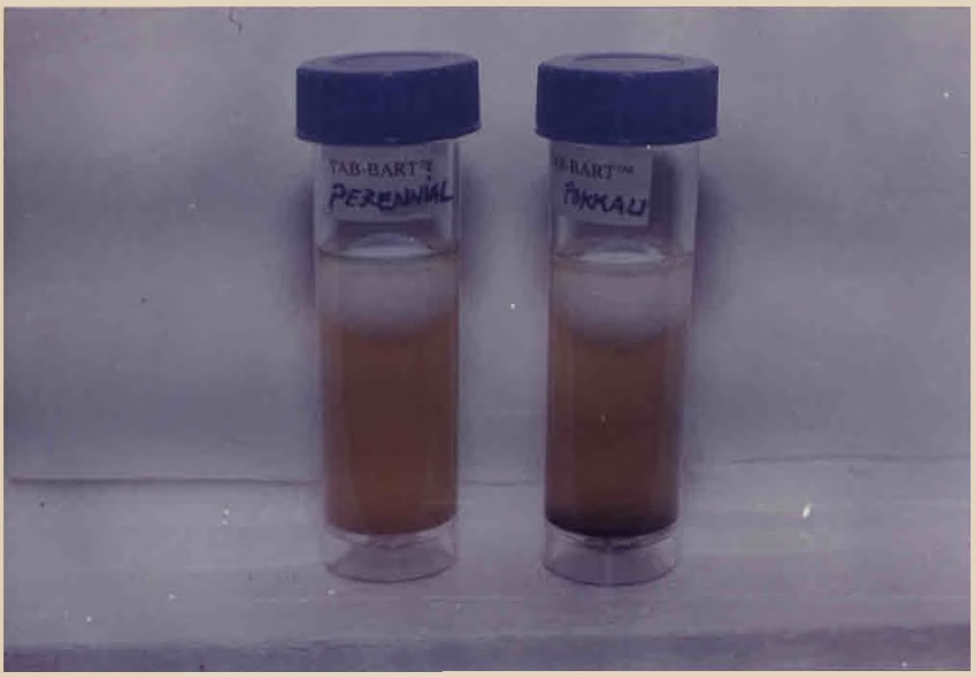

TAB-BART™
PERENNIAL

PLATE V WATER SAMPLES INCORPORATED WITH TRIPHENYL TETRAZOLIUM CHLORIDE (POKKALI & PERENNIAL)

PLATE VI SEDIMENT SAMPLES INCORPORATED WITH TETRAZOLIUM CHLORIDE (POKKALI & PERENNIAL)

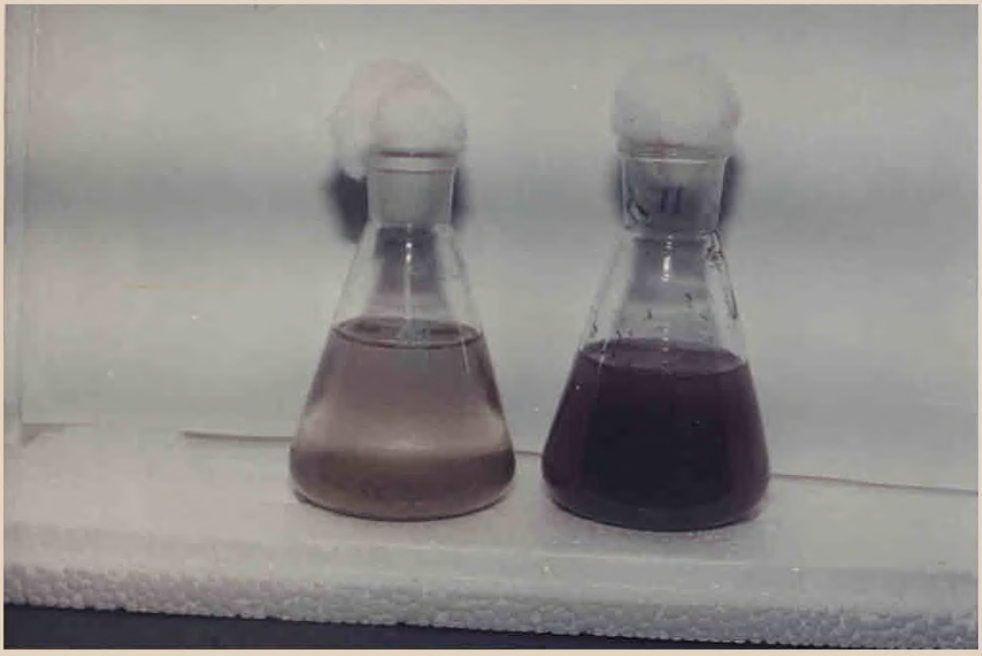


PLATE VII COLONIES IN SEAWATER AGAR MEDIUM AFTER ADDITION OF TRIPHENYL TETRAZOLIUM CHLORIDE (TTC)

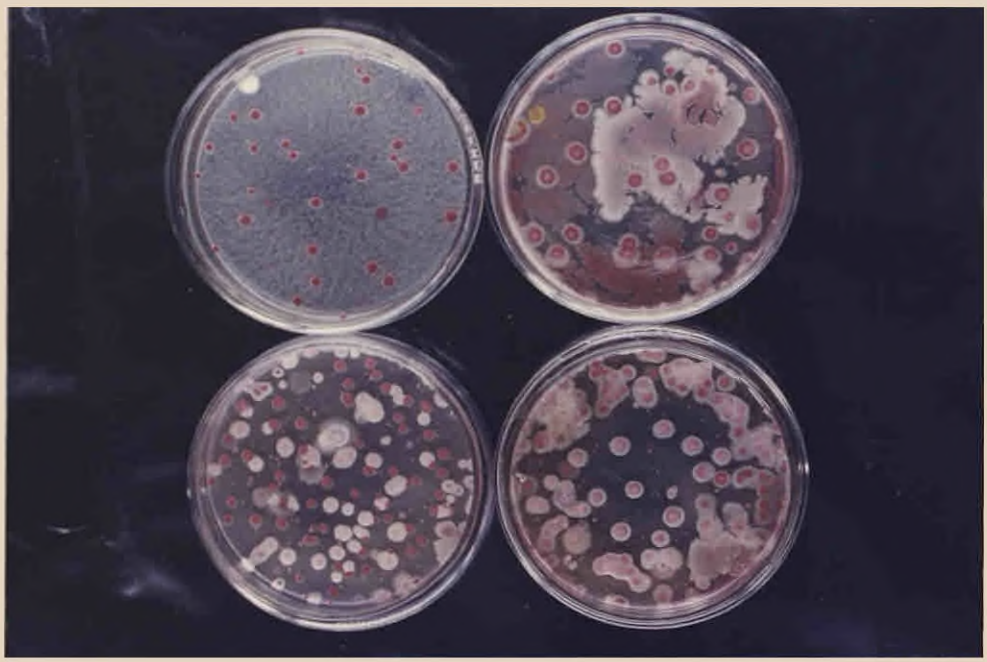


PLATE VIII A. FUNGAL COLONIES

PLATE VIII B. FUNGAL COLONIES

PLATE VIII C. FUNGAL COLONIES

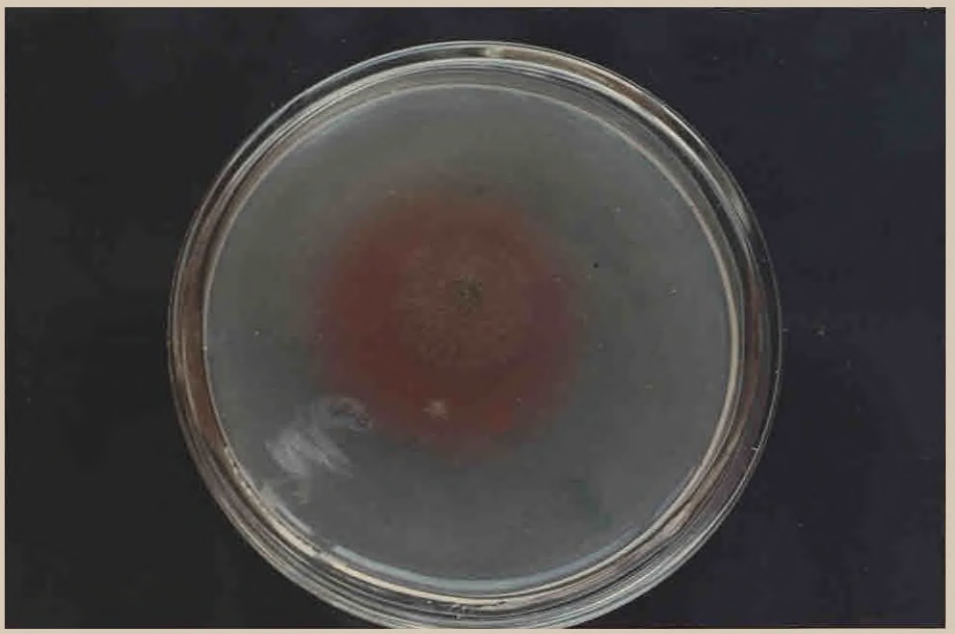
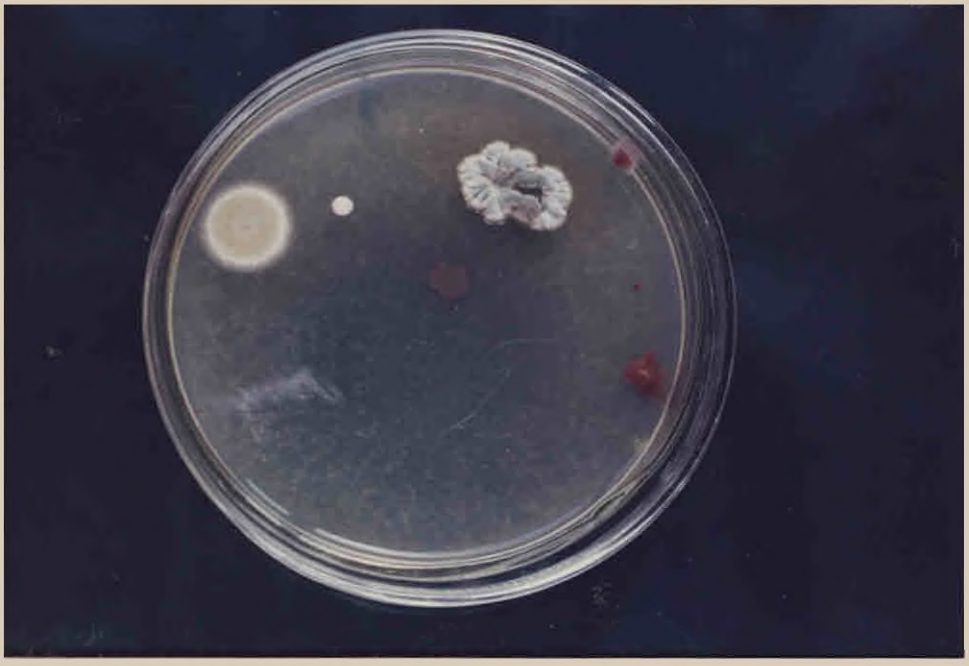


PLATE VIII D. FUNGAL COLONIES

PLATE VIII E. FUNGAL COLONIES



tubes were observed every hour and the medium started changing the colour after 4 or 5 hours. Both the samples harboured somewhat equal intensity of bacteria showing fertility of the water; but Pokkali field surface water contained more hydrogen sulphide producing anaerobic microbes, as more black pigments were recorded in Pokkali field surface water inoculated tubes, indicating the presence of rich sulphate ions in sediment (Plates III & IV).

Addition of TTC turned the samples, especially those from Pokkali fields, red in colour, which indicated the fertility in the sample (Plates V and VI). Colonies obtained in media containing TTC are shown in Plate VII. Also the media were found to be yielding profuse growth of fungi of genera *Penicillium, Aspergillus, Rhizopus* etc. (Plate VIII a, b, c, d, e)

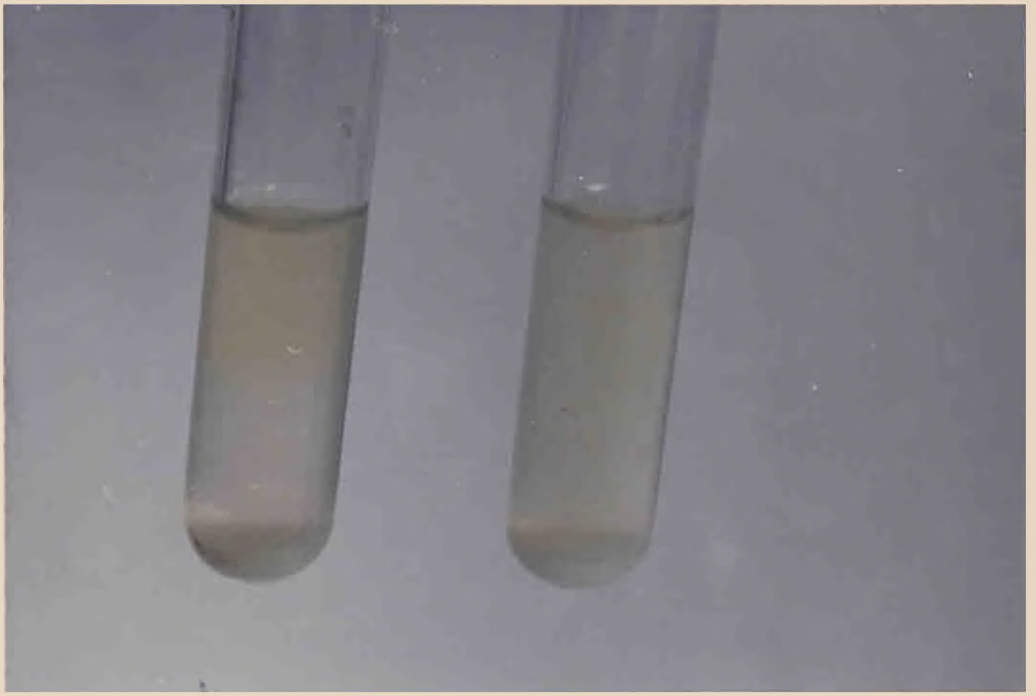
QUALITATIVE ANALYSIS

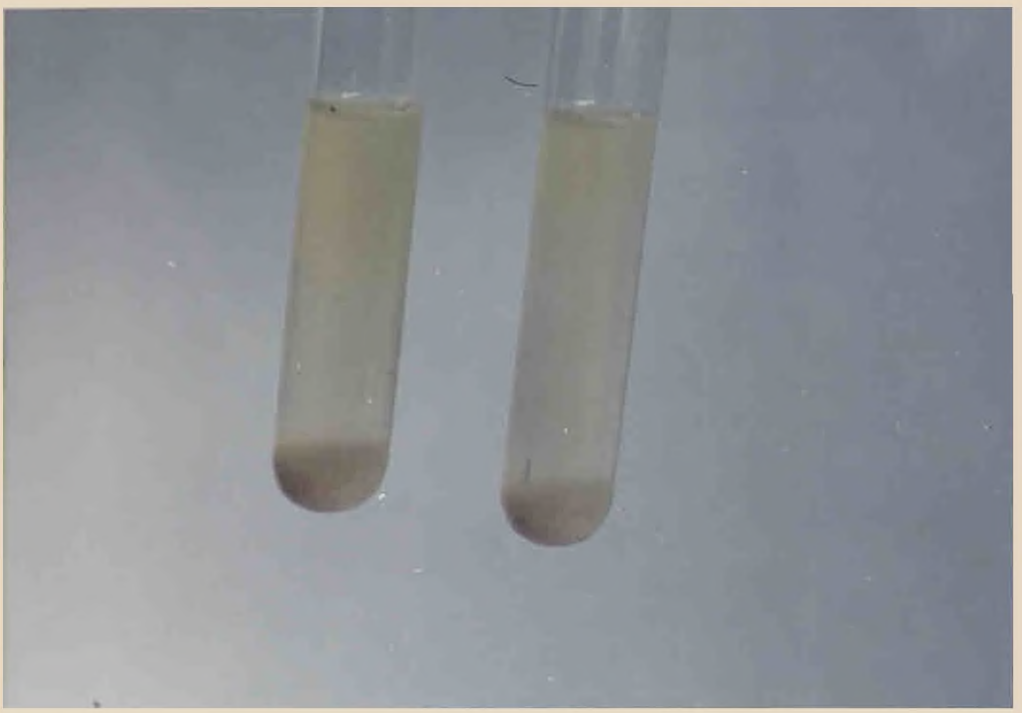
Altogether 31 strains of gram negative rods were isolated on initial screening with oxidase positive and glucose negative nature of the isolates and identified into three genera. Oxidase - positive aerobes immediately formed purple colour products when their cells were mixed with a solution of N'N'N - paraphenyl diamine dihydrochloride or a related oxidizable amine. The reaction is associated with the presence of a cytochrome of the C type in the transport chain.

PLATE IX FLUORESCENCE UNDER ULTRA-VIOLET LIGHT (SLANTS)

PLATE X (a) FLUORESCENCE IN BROTH CULTURE

PLATE X (b) FLUORESCENCE IN BROTH CULTURE





Cytochromes in bacteria

In bacteria the cytochromes are normally located in the cell membrane and vary from one species to another, and sometimes even in a single species, under different cultural conditions. Cytochromes appear to be absent from some facultative and obligate anaerobes. In bacteria, a variety of cytochromes may act as terminal oxidases. More than one oxidase may be present in a given species: a, d and o. P. *putida* contains a soluble P-450 which effects hydroxylation of camphor and related compounds in a cyclic reaction sequence. Some bacteria contain modified C-type cytochromes, but differ chemically and spectroscopically from normal C-type cytochromes. Cytochromes contain haem a- which carries one formyl group and one long hydrophobic side chain.

Out of the 13 isolates 11, were identified as pathogenic *Pseudomonas* based on their bio-chemical activities. Three were giving yellowish green Fluorescent pigment. (Plates IX, Xa, Xb). Almost all the isolates were asporogenous Gram-Negative short rods, usually somewhat pleomorphic in nature. All the 31 isolates were actively motile.

Table V illustrates the activity of the 31 isolates in Hugh and Leifson's glucose medium. Glucose was

Table V

SUGAR FERMENTATION IN
HUGH & LEIFSON'S GLUCOSE MEDIUM

ORGANISM (CULTURE No.)	OXIDATIVE	FERMENTATIVE	ALKALINE	NO REACTION
1.	+	-	-	-
2.	-	+	-	-
3.	+	-	-	-
4.	+	-	-	-
5.	+	-	-	-
6.	+	-	-	-
7.	-	-	+	-
8.	+	-	-	-
9.	+	-	-	-
10.	-	+	-	-
11.	-	+	-	-
12.	-	+	-	-
13.	-	+	-	-
14.	-	+	-	-
15.	-	+	-	-
16.	-	+	-	-
17.	-	+	-	-
18.	-	-	+	-
19.	-	+	-	-
20.	-	+	-	-
21.	-	+	-	-
22.	-	+	-	-
23.	-	+	-	-
24.	-	+	-	-
25.	-	-	-	+
26.	-	-	-	+
27.	-	+	-	-
28.	-	-	+	-
29.	-	-	+	-
30.	-	+	-	-
31.	-	+	-	-

Table VI

HYDROGEN SULPHIDE PRODUCTION IN CYSTINE MEDIUM

Organism	H_2S Production
Pseudomonas putida	-
P. putida	+
P. putida	-
P. fluorescens	-
Alcaligenes faecalis	+
P. putida	-
P. fluorescens	+
P. anguillisieptica	-
Alteromones piscida	+
P. fluorescene	-
P. putida	-
P. fluorescence	+
P. fluorescence	+

utilised oxidatively by 22.58% of the isolates which belonged to *Pseudomonas* group I and group II and 54.83% were exhibiting fermentative type of metabolism. 12.9% recorded, alkaline reaction forming *Pseudomonas* group III and 6.45% gave no reaction to the substrate and came under *Pseudomonas* Group IV (Table III). No reaction in the two tubes indicated that the particular carbohydrate is not attacked by the test organisms.

L-cysteine medium was inoculated to test the hydrogen sulphide production by the test organisms and incubated for seven days at room temperature. Six of the pathogenic pseudomonads produced hydrogen sulphide indicated by the blackening of lead acetate paper kept in the medium (Table VI)

Utilization of some nitrogenous compounds were tested, and the results are as follows. (Table VII) The nitrogen requirements of, most marine bacteria are satisfied either by ammonium salts or amino acids. In the present study, 5 organic compounds and 2 inorganic compounds were used to detect the ability of 13 isolates to metabolise them. Most of the cultures grew luxuriantly in inorganic salts like ammonium chloride when compared to organic compounds. When urea was used as the substrate, 61.53% were capable of reducing urea, while 76.92% of the isolates were found to produce ammonia. When asparagine was used as the substrate, 15.38% and 76.92% reduced the substrate to

Table VII

UTILIZATION OF DIFFERENT NITROGENOUS COMPOUNDS IN GLUCOSE MEDIA BY 13 ISOLATES

SUBSTRATE	ORGANISM	GROWTH & TURBIDITY	NITRATE REDUCTION	AMMONIA PRODUCTION
UREA	*Pseudomonas putida*	+	-	+
	P. putida	+	-	+
	P. putida	+ Sediment	+	-
	P. fluorescens	+	+	+
	Alcaligens faecalis	+ Sediment	-	-
	P. putida	+	+	+
	P. fluorescens	++	+	+
	P. anguillise-eptica	+	+	-
	Alteromonas piscida	+ Sediment	+	+
	P. fluorescens	+(Pellicle)	-	+
	P. putida	+	+	+
	P. fluorescens	++	+	+
	P. fluorescens	++ Pellicle	-	+
ASPARAGINE	*Pseudomonas putida*	++	-	+
	P. putida	++	+	+
	P. putida	+	-	+

SUBSTRATE	ORGANISM	GROWTH & TURBIDITY	NITRATE REDUCTION	AMMONIA PRODUCTION
	P. fluorescens	+ Pellicle	-	-
	Alcaligenes faecalis	+	-	+
	P. putida	+ Pellicle	-	-
	P. fluorescens	+	-	+
	P. anguillisc-eptica	+ Pellicle	-	+
	Alteromonas piscida	++ Pellicle	-	+
	P. fluorescens	++ Pellicle	-	+
	P. putida	+ Sediment	-	-
	P. fluorescens	++ Pellicle	-	+
	P. fluorescens	+	+	+
AMMONIUM OXALATE	*Pseudomonas putida*	+ Sediment	-	+
	P. putida	+	-	+
	P. putida	+ Sediment	-	+
	P. fluorescens	+ pellicle	-	+
	Alcaligenes faecalis	+ Sediment	-	+
	P. putida	+ Sediment	-	+
	P. fluorescens	+ Sediment	-	+
	P. anguillisc-eptica	+ Sediment	-	+

SUBSTRATE	ORGANISM	GROWTH & TURBIDITY	NITRATE REDUCTION	AMMONIA PRODUCTION
	Alteromonas piscida	+ Sediment	-	+
	P. fluorescens	+	-	+
	P. putida	+ Sediment	-	+
	P. fluorescens	+ Sediment	+	+
	P. fluorescens	+ Sediment	-	+
ANILINE	*Pseudomonas putida*	+	-	-
	P. putida	+	+	-
	P. putida	+	-	-
	P. fluorescens	+	-	+
	Alcaligenes faecalis	+ Sediment	-	-
	P. putida	+	-	-
	P. fluorescens	+	-	-
	P. anguillsc-eptica	+	-	-
	Alteromonas piscida	+	-	+
	P. fluorescens		-	-
	P. putida	+ Sediment	-	+
	P. fluorescens	+	+	+
	P. fluorescens	+	-	+

SUBSTRATE	ORGANISM	GROWTH & TURBIDITY	NITRATE REDUCTION	AMMONIA PRODUCTION
CYSTINE	*Pseudomonas putida*	+	+	+
	P. putida	+	+	+
	P. putida	+ Pellicle	+	+
	P. fluorescens	+	+	+
	Alcaligenes faecalis	+ Sediment	+	+
	P. putida	+	-	+
	P. fluorescens	+	+	+
	P. anguillsceptica	Pellicle	-	+
	Alteromonas piscida	+ Pellicle	-	+
	P. fluorescens	+ Pellicle	+	+
	P. putida	Sediment	-	-
	P. fluorescens	++Pellicle	-	-
	P. fluorescens	++	-	-
GLUTAMIC ACID	*Pseudomonas putida*	+ Pellicle	+	+
	P. putida	+ Pellicle	+	+
	P. putida	+ Sediment	-	+
	P. fluorescens	+	-	-
	Alcaligenes faecalis	Slightly turbid	-	-
	P. putida	+	-	+

SUBSTRATE	ORGANISM	GROWTH & TURBIDITY	NITRATE REDUCTION	AMMONIA PRODUCTION
	P. fluorescens	-	-	+
	P. anguillsceptica	+ Pellicle	-	-
	Alteromonas piscida	+ Pellicle	-	-
	P. fluorescens	+ Pellicle	+	+
	P. putida	+ Sediment	+	-
	P. fluorescens	+ Sediment	Slightly +	-
	P. fluorescens	++Pellicle	-	+
AMMONIUM CHLORIDE	*Pseudomonas putida*	+	+	+
	P. putida	+	-	+
	P. putida	+ Pellicle	+	+
	P. fluorescens	+	+	+
	Alcaligenes faecalis	Sediment	+	+
	P. putida	+	-	+
	P. fluorescens	++	+	+
	P. anguillsceptica	+	-	+
	Alteromonas piscida	+ Pellicle	++	+
	P. fluorescens	+	+	+
	P. putida	+ Sediment	+	+
	P. fluorescens	+ Pellicle	-	+
	P. fluorescens	+ Pellicle	+	+

nitrite and ammonia respectively. Out of the 13 isolates 53.84% were found to utilise the substrates, while 76.92% were shown to harbour ammonia producing enzymes when cystine was given as the substrate. 38.46% were able to produce nitrite and 53.84% were found to produce ammonia when glutamic acid was given as the substrate. When ammonium chloride was used as the substrate, 69.23% were found to reduce nitrogen, while all the isolates were found to produce ammonia. In the case of ammonium oxalate, only one isolate (7.69%) was able to reduce nitrate, but all were found to produce ammonia. When aniline was used as the nitrogenous substrate, 15.38% were found to metabolise efficiently, whereas 38.46% could produce ammonia. In general, ammonia production was the domineering reaction, which was at its maximum, in two substrates like ammonium chloride and ammonium oxalate (100% in both). The reducing ability was recorded high when ammonium chloride was used as a substrate where 69.2% of the isolates were found to be positive. This effect was found to be as low as 7.6% when ammonium oxalate was the substrate given. The highest percentage of isolates producing nitrite and ammonia for a single substrate was recorded for ammonium chloride, where 69.23% isolates were positive for both the tests.

Growth characteristics at $5^{o}C$ and $37^{o}C$ were tested and Table VIII illustrates the results obtained from this experiment. This test is very essential for species identification of oxidase positive gram-negative non-

Table VIII

GROWTH CHARACTERISTICS AT $5^{o}C$ and $37^{o}C$

Organism (Culture No)	$5^{o}C$	$37^{o}C$
Pseudomonas putida	good growth, No fluorescence	moderate growth; No fluorescence
P. putida	Moderate growth	affluent growth; No fluorescence
P. putida	Poor growth	good growth; No fluorescence
P. fluorescens	poor growth	good growth and fluorescence
Alcaligenes faecalis	poor growth	good growth
P. putida	Moderate growth No fluorescence	good growth; No fluorescence
P. fluorescens	Coccoid type of growth No fluorescence	growth present; No fluorescence
P. anguillisc-eptica	No much growth	good growth and fluorescence
Alteromonas piscida	No growth	good growth; No fluorescence
P. fluorescens	Moderate growth No fluorescence	good growth and fluorescence
P. putida	poor growth	good growth; No fluorescence
P. fluorescens	poor growth	good growth; No fluorescence
P. fluorescens	Moderate growth	Moderate growth;

Growth at different concentrations of sodium chloride

Organism	at 0 % Nacl	at 5 % Nacl	at 7 % Nacl	at 10 % Nacl
Pseudomonas putida	+	+		
P. putida	+	+		
P. putida				
P. fluorescens	++	+		
Alcaligenes faecalis				
P. putida	++	+		
P. fluorescens	+	++	+	
P. anguilliseptica	++	+		
Alteromonas piscida	+	+		
P. fluorescens	++	+		
P. putida	+			
P. fluorescens	++	+		
P. fluorescens	++	+		

fermentative rods. Out of the 13 oxidase positive non-fermentative gram-negative rods, 38.46% exhibited moderate growth and 38.46% exhibited poor growth, and 15.38% exhibited no growth at all at 5°C. At 37°C, 76.92% exhibited affluent growth and 23.07% exhibited moderate growth. All the three green fluorescent Pseudomonads exhibited very good growth at 37°C, whereas at 5°C they exhibited very poor growth.

Table IX shows the growth at different concentrations of sodium chloride. At 0% sodium chloride 46.15% exhibited affluent growth, 38.46% exhibited normal growth and 15.38% exhibited no growth at all. At 5% concentration of sodium chloride, only one isolate exhibited affluent growth, out of the 13; 69.23% exhibited normal growth, while 23.07% exhibited no growth. At 7% sodium chloride, only one isolate was giving good growth, which was identified as *Alteromonas piscida*. None of the isolates were capable of growing at 10% sodium chloride. All the isolates, capable of growing at 0% and 5% sodium chloride were designated as *P. florescence* and *P. putida*. Isolates incapable of growing at higher concentrations of sodium chloride are designated as *P. anguillisceptica*.

Table X illustrates the ability of the isolates to use citrate as the sole carbon source. The incubation was carried out at the optimum growth temperature of the

Table X

UTILIZATION OF CITRATE AS CARBON SOURCE
(SIMMON'S CITRATE AGAR)

Organism	Result
Pseudomonas putida	+
P. putida	+
P. putida	-
P. fluorescens	+
Alcaligenes faecalis	-
P. putida	+
P. fluorescens	-
P. anguillisceptica	+
Alteromones piscida	-
P. fluorescens	+
P. putida	+
P. fluorescens	+
P. fluorescens	+

Table XI

PROTEOLYTIC AND AMYLOLYTIC ACTIVITY OF PATHOGENIC PSEUDOMONADS

Organism	Gelatin	Caesein	Starch	Arginine
Pseudomonas putida	-	-	-	+
P. putida	-	-	-	+
P. putida	-	-	-	+
P. fluorescens	+	+	-	+
Alcaligenes faecalis	-	-	-	-
P. putida	-	-	-	+
P. fluorescens	+	+	-	+
P. anguillisceptica	+	+	-	-
Alteromonas piscida	+	+	+	-
P. fluorescens	+	+	-	+
P. putida	+	-	-	+
P. fluorescens	+	+	-	+
P. fluorescens	-	+	-	+

Table XII

SPECIES IDENTIFICATION TESTS FOR IDENTIFICATION OF PSEUDOMONAS Sp.

Culture No.	Growth at Different Concentrations of Sodium Chloride				Gelatin Liquification	Fluorescence	Caesein	Starch hydrolysis	Arginine	Citrate as carbon source	Identified Genera
	at 0% NaCl	at 5% NaCl	at 7% NaCl	at 10% NaCl							
1.	+	+	-	-	-	-	-	-	+	+	Pseudomonas putida
3.	+	+	-	-	-	-	-	-	+	+	Pseudomonas putida
4.	-	-	-	-	-	-	-	-	+	-	Pseudomonas putida
5.	++	+	-	-	+	+	+	-	+	+	Pseudomonas fluorescens
6.	-	-	-	-	-	-	-	-	-	-	Alcaligenes faecalis
7.	++	+	-	-	-	-	-	-	+	+	Pseudomonas putida
8.	+	++	+	-	+	+	+	-	+	-	Pseudomonas fluorescens
9.	++	+	-	-	+	-	+	-	-	+	Pseudomonas anquillisceptica
18.	+	+	-	-	+	-	+	+	-	-	Alteromonas piscida
25.	++	+	-	-	+	+	+	-	+	+	Pseudomonas fluorescens
26.	+	-	-	-	-	-	-	-	+	+	Pseudomonas putida
28.	++	+	-	-	+	-	+	-	+	+	Pseudomonas fluorescens
29.	++	+	-	-	+	-	+	-	+	+	Pseudomonas fluorescens

Table XIII

PERCENTAGE OF COMPOSITION OF THE
13 ISOLATES FROM POKKALI AND
PERENNIAL FIELDS ISOLATED DURING MAY AND JUNE '97.

Species	Nos. Obtained	Percentage
Alteromonas piscida	1	7.69%
Pseudomonas putida	5	38.46%
Pseudomonas fluorescens	5	38.46%
P. anguillisceptica	1	7.69%
Alcaligenes faecalis	1	7.69%

PLATE XI STARCH HYDROLYSIS

pathogenic Pseudomonads and 69.23% were capable of using citrate as the carbon source.

Proteolytic activity was tested by using gelatin, caesein and arginine (Table XI). Only seven isolates (53.84%) were found to liquify gelatin, which included *P. fluorescens P. anguillisceptica Alteromonas piscida*. 53.84% were hydrolysing caesein and 76.92% fermented arginine. Species like *P. fluorescences* and *P. putida* were very actively hydrolysing arginine, whereas *Alcaligenes faecalis*, *Alteromonas piscida* and *P. anguillisceptica* were unable to hydrolyse arginine. Amylolytic activity was very poor among the isolates and 92.3% were not hydrolysing starch. Only *Alteromonas piscida* was found to be capable of hydrolysing starch. (Plate XI)

The different species and their percentages obtained based on the above tests are given in Table XII & XIII.

ANTIBIOTIC SENSITIVITY

In the present study sensitivity towards Penicillin, Kanamycin and Ampicillin was tested (Table XIV) and all the isolates were found to be resistant to penicillin (15 mcg/disc) Resistance to a particular antibiotic is often associated with resistance to other chemically related antibiotics. Usually such a cross

Table XIV

COMPARATIVE SUSCEPTIBILITIES STO ANTIBIOTICS
BY PATHOGENIC *PSEUDOMONAS*

Organism	Pencillin (15 mcg/ disc)	Kanamycin (30 mcg/ disc)	Ampicillin (10 mcg/ disc)
Pseudomonas putida	-	+	-
P. putida	-	+	-
P. putida	-	-	-
P. fluorescens	-	-	-
Alcaligenes faecalis	-	+	-
P. putida	-	-	-
P. fluorescens	-	+	-
P. anguillisceptica	-	+	-
Alteromones piscida	-	+	-
P. fluorescens	-	-	-
P. putida	-	-	-
P. fluorescens	-	-	-
P. fluorescens	-	-	-

PLATE XII ANTAGONISTIC ACTIVITY

A. POSITIVE IN ALL WELLS

B. NEGATIVE IN ALL WELLS

PLATE XIII ANTAGONISTIC ACTIVITY

A. POSITIVIE IN TWO WELLS

B. NEGATIVE IN TWO WELLS

resistance occur only among the sulphonamides and so in the present study it was seen that Ampicillin (10 mcg/disc), which is chemically related to Penicillin was also found to be resistant by the cultures. Resistance occurred as a result of single mutation, is commonly sensitive against a single antibiotic or a group of cross resistant antibiotics. 46.15% were sensitive to Kanamycin (30 mcg/disc) which is a broad spectrum amino glycoside antibiotic.

ANTAGONISTIC ACTIVITY

Well plate method was used to test the antagonistic effect of pathogenic Pseudomonads against some test pathogens (Table XV). 38.76% were very actively inhibiting *Vibrio anguillarum* H^{10} and 53.84% were actively inhibiting *Cytophaga* 46.15% were very actively inhibiting *Edwardsiella* and *Mycobacterium* sp. (Plates XIIa, XIIb, XIIIa, XIIIb)

DISCUSSION

The principal interest of the investigation was to isolate pathogenic *Pseudomonas* spp. and to record their occurrence and distribution in water and sediment samples from Perennial and Pokkali fields. Totally three observations were made in this study, along with which environmental parameters were studied. Detailed biochemical analysis had been done, and importance was given to sensitivity towards antibiotics. Also, as *Pseudomonas* are well known for their antagonistic activity, and as there is a growing concern in the study of antagonistic compounds, all the 13 isolates were tested against pathogens by cross steaking and well plate method. As aquaculture ponds are unexplored area for antagonistic activity of pathogenic Pseudomonads, this study on ecophysiology of pathogenic *Pseudomonas* spp. was intended to monitor total bacterial flora and pathogenic *Pseudomonas* in Seawater Agar medium and Pseudomonas Agar.

Pseudomonds are opportunistic pathogens. Whenever they are not parasitic, they will opt for heterotrophic type of nutrition. Their important function in the pond may be denitrification Gran (1901) classified bacteria into four categories according to their action on nitrate and nitrite.

1. Both nitrate and nitrite reduced to nitrogen.
2. Nitrate reduced to nitrite and ammonia.

3. Nitrite but not nitrate reduced.
4. Neither nitrate nor nitrite reduced.

Some other nitrate and nitrite reducing bacteria are now recognised. The term denitrification is applied exclusively to the liberation of free nitrogen. So, considering all these factors, seven nitrogenous compounds were tested with isolated pathogenic Pseudomonads to estimate the denitrification potential of *Pseudomonas*.

The discussion is presented in the following headings.

- Quantitative & qualitative distribution of bacterial flora.
- Relationship of environmental parameters with microflora.
- Fluorescent pigment production and biochemical activities of Pseudomonads.
- Bacterial denitrification.
- Sodium chloride tolerance.
- Antibiotic susceptibility.
- Antagonistic activity.

In the present study, water and sediment samples were plated with two different selective media, apart from seawater agar mentioned in the results, for enumeration of

pathogenic Pseudomonads. It is always better to employ more than one medium to estimate the qualitative and quantitative distribution of a single group of bacteria and the samples were enriched with malachite green broth before pour plating procedures (Table II). However for detecting antibiotic sensitivity and antagonistic activity against test pathogen only SWA medium was used. Pseudomonas agar with Triphenyl Tetrazolium Chloride (TTC) was found best for pathogenic *Pseudomonas* isolates. Cetrimide agar supplemented with TTC gave very poor results. Of the three media used for isolation of pathogenic *Pseudomonas*, Pseudomonas agar supplemented with TTC gave best results for estimation of fungal population (Plate VIII) also.

As the water and sediment samples were from brackishwater area 50% aged seawater was employed for media preparation.

Results of Bio-detector medium TAB BART™ showed total aerobic bacteria and anaerobes were more in Pokkali field when compared to Perennial pond indicating the fertility of Pokkali field when compared to perennial pond. (Plates III and IV)

Studies by Velammal (1994) have shown that *Pseudomonas aeruginosa* occurred in high numbers in water, sediment and finfish samples during postmonsoon seasons. (January- March) in Pondicherry coastal waters, and could

not be detected during premonsoon season (August-September) and rarely during monsoon (October). It was also isolated from sundried and salted fish. But in the present study, conducted in early monsoon and monsoon period, high numbers of *Pseudomonas* occurred in the selective medium. Maximum bacterial populations were observed during monsoon months. The primary environmental factors influencing the pathogenic Pseudomonas include moisture, temperature, acidity, organic matter and inorganic matter supplied (Chandrika 1984). The influence of seasons occurred only for the combination of primary determinants.

45 heterotrophic bacterial strains were isolated from Kavarathi and Minicoy islands from sea water and their morphological and cultural characteristics were studied. Predominant bacteria was *Pseudomonas* and all the 15 were denitrifiers and 7 of them produced greenish fluorescent pigment (Chandrika 1989)

The prevalence of *Pseudomonas* has been demonstrated in both perennial and Pokkali fields in the present study. Values in excess of 1000 ml and 10000 gm were not uncommon (Figure II, III, IV, V). The physiology and metabolism of the potential microbial group has been the subject of considerations as it cause diseases of plant and animals in the environment. *Pseudomonas* can grow readily in ordinary medium and very rarely it will need growth factors

for development. 10% of the isolates will need amino acids and vitamins, 30% will require complex mixture of growth factors (Alexander, 1984). In sediment, Pseudomonas occurred at levels of 15-20%. At times, they will be facultatively anaerobic and reduce iron in anaerobic condition. Also they can synthesize phytase enzyme and convert elemental sulphur or thiosulphate to sulphate. They are capable of liberating hydrogen sulphide also. In the present study, gelatin and caesein were liquefied by 53.84% of cultures and Arginine by 76.92% , whereas starch was poorly hydrolyzed (Plate XI). Proteolytic activity was very high when compared to amylolytic activity. (Table XI)

Only two or three types of fungi like *Pencillium*, *Aspergillus* and *Rhizopus* were observed in the culture ponds in the first observation (Plates VIII a, b, c, d & e). It has been found out by Alexander (1978) that the presence of one or more group of fungi is frequently related to the vegetation. In the present case, mangroves plants like *Avicinnia* and *Rhizophora* were the vegetation in the study area.

The production of fluorescent pigment was there in three strains and other Pseudomonas isolates were non-pigmented (Plate IX, Xa and Xb). It is well known that, fluorescent pigment production property is unstable and dependent on the nature of the medium for its manifestation (Seyleene and Starck (1943)). The fluorescein pigment

production property was tested with ingredients like dipotassium hydrogen phosphate, glycerol and magnesium chloride and the manifestation was more in dipotassium hydrogen phosphate.

All the thirteen cultures were gram-negative short rods which exhibited pleomorphism. Pleomorphism may be exhibited as chain formation, a faint gram negative reaction and also by filament formation. Individual isolates showed minor variations such as lack of pellicle formation, slight intense pigmentation and dull or rough surface appearance, difference in opacity of growth on solid medium, Suire (1958) has described the production of spontaneous nutrients by *Pseudomonas*.

Six of the pathogenic Pseudomonads were capable of hydrogen sulphide production indicating intensive biochemical capacity in producing H_2S (Table VI)

All were motile and most of the isolates grew well between $5^{o}C$ and $37^{o}C$ with a low exceptions (Table VIII). Out of the 13 cultures, 38.46% exhibited moderate growth and another 38.46% exhibited poor growth and 15.38% exhibited no growth at $5^{o}C$. At $37^{o}C$, 76.92% exhibited affluent growth and 23.07% exhibited moderate growth.

Growth at lower temperature was found to be an identification factor, as poor growth in $5^{o}C$ indicated *P. fluorescence, P. putida, and P. anguillisceptica*. But

Alteromonas piscida was unable to grow at 5°C. Growth at 37°C was there for *P. fluorescence* and *P. putida* but *P. anguillisceptica* was unable to grow at 37°C (Table VIII). So it is concluded that the growth temperature alone can't be used for the polarly flagellated Gram negative rods, because of the normally wide temperature range. The elevated temperature test, at 37°C must be interpreted in conjunction with other characters. Haynes (1951) stated that generally good growth was obtained at 37°C when compared to 5°C.

Utilisation of different nitrogenous compounds in glucose media was tested and in hydrolysis of urea, 61.53% was capable of reducing urea to nitrite, while 76.92% were capable of producing ammonia. All the isolates developed good growth in the urea incorporated peptone broth (Table VII). DeTurk (1955) reported the adaptive formation of urease in washed suspensions of *Pseudomonas* spp.

When asparagine was tested as the substrate, 15.38% and 76.92% reduced the substrate to nitrite and to ammonia respectively. The high percentage of ammonia production showed the proteolytic activity of *Pseudomonas* spp.(Table VII). When aniline was used 15.36% was found to be showing nitrate reduction, while 38.46% could produce ammonia.

Nitrate forming bacteria are somewhat more difficult to demonstrate than the nitrite forming organism (Waksman, 1933). Although denitrifiers are usually

distributed in the aquatic environment as pure cultures, only 5% of the bacterial species are endowed with the ability to liberate free nitrogen from nitrate or nitrite in the presence or abundance of organic matter (Zobell, 1946). 34 species of marine bacteria studied by Zobell and Upham (1944) reduced nitrate to nitrite in enrichment media. Most of the *Bacillus, Achromobacter, Pseudomonas, Serratia* and *Vibrio* are extremely active nitrate reducers. *Pseudomonas azotogena* and *P. perfectomarinus* are the only organisms among the 60 species described by Zobell and Upham (1944) to reduce the nitrate to free nitrogen. 38.46% of the isolates were capable of reducing nitrate to free nitrogen. Ammonification and peptonisation are mainly done by Pseudomonadales and Eubacteriales. There is hydrographical evidence that ammonia and nitrite are produced only in the bottom sediment, as it is an anaerobic phenomenon, whereas most ammonification occurs only in surface water (Zobell, 1946).

53.84% of the isolates were found to reduce the sulphur containing amino acid L. cystine, while 76.92% were shown to have ammonia producing enzymes, when cystine was used as the nitrogenous substrate (Table VII). When gluctamic acid was used as the substrate, 38.46% were able to produce nitrite and 53.84% were found to produce ammonia. The nitrogen requirements of most of the bacterial flora are satisfied either by amino acids or ammonium salts. When ammonium chloride was used as the substrate 69.23% were able

to produce nitrite, while, all the isolates were found to be producing ammonia. When ammonium oxalate was used as the substrate, only one isolate was found to reduce the compound to nitrate to nitrite, but all (100%) were capable of producing ammonia (Table VII)

In the present study, 100% production of ammonia was noticed from inorganic salts like ammonium chloride and ammonium oxalate; in pathogenic *Pseudomonas* spp. when compared to organic compounds or amino acids. The number of cultures, which grew in glucose media containing these substrates are shown in Table VII.

It is also noted that, Pseudomonas, the veteran degrader of organic matter in the marine eco-system dominated the pre-monsoon flora during 1974-75 (Chandrika 1984).

Most of the isolates were found to be growing well in peptone glucose water without sodium chloride and in media containing 5% sodium chloride; but affluent growth was there in medium without sodium chloride, while only average growth was observed in media with 5% sodium chloride. At 7% concentration and 10% concentration of sodium chloride, growth was absent. In sodium chloride broth culture, a dense slimy mass accumulated at the base of the tube, leaving the supernatant very clear. Another feature was the absence of fluorescence in the medium (Table IX), when sodium chloride was incorporated in the medium. Citrate was found to be used

by 69.23% of the 13 isolates as the sole carbon source for growth as shown in Table X.

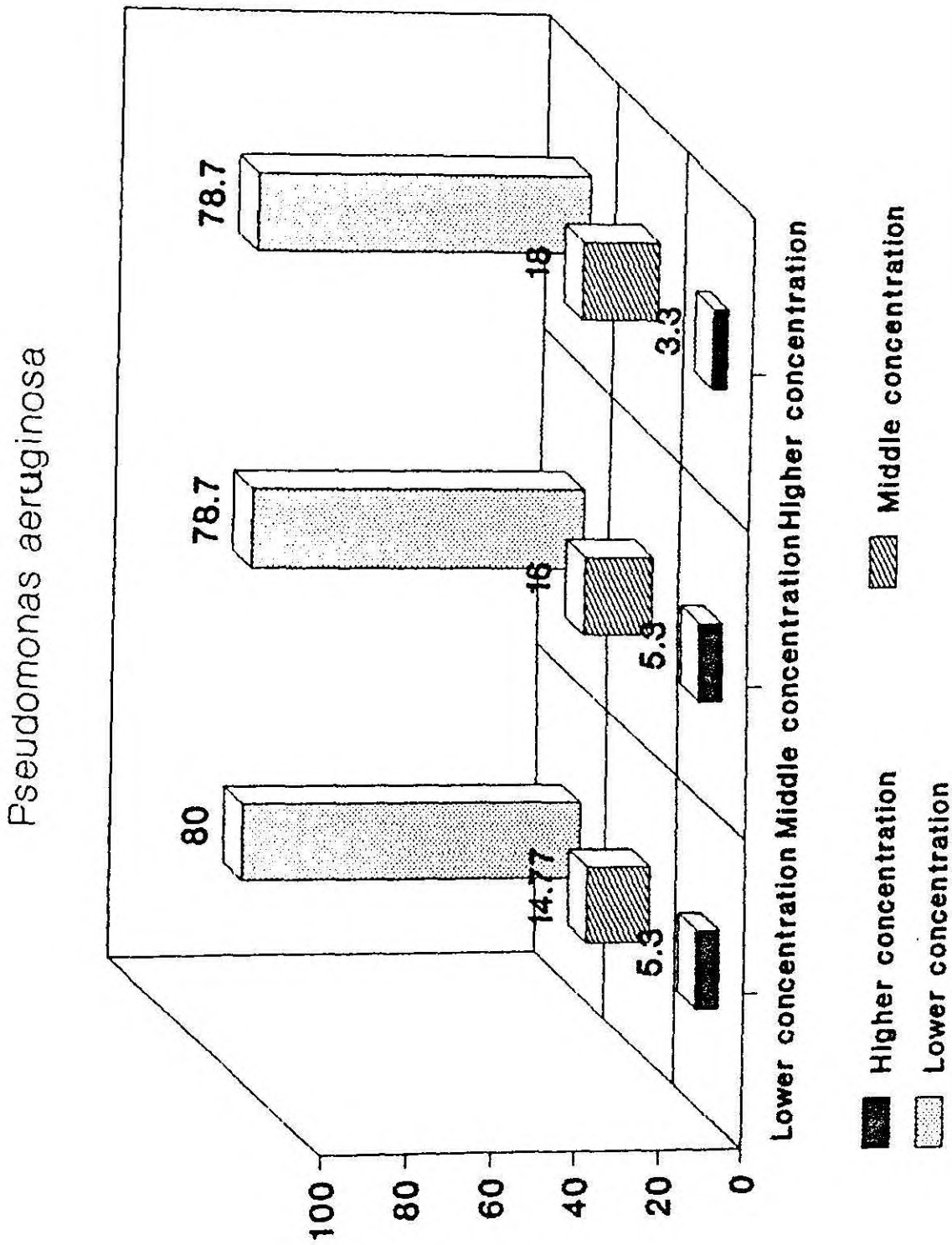
As already stated proteolytic activity and amylolytic activity of the isolates were tested and results showed that 53.84% of the isolates liquified gelatin, and 76.92% fermented Arginine. Amylolytic activity was poor, since only one estate, was hydrolysed starch (Plate XI) caesin was hydrolysed by 53.84% of the isolates. These tests formed important criteria in identifying the species (Table XI).

The overall biochemical tests, used for identification upto species level is given in Table No. XII.

Three antibiotics were subjected to sensitivity tests and none of the cultures were found to be sensitive to penicillin (15mcg/disc). Out of the 13 cultures tested, five were found to be sensitive to Kanamycin (30 mcg /disc), whereas all the isolates were resistant to ampicillin (10 mcg/disc). The resistance of Pseudomonas against various antibiotics are well known if the origin of the culture is from human beings. As the cultures in the present study were isolated from water and sediment samples, Kanamycin and Ampicillin were so effective in inhibiting the growth of *Pseudomonas* in synthetic media. (Table XIV)

In all the antibiotics tested, out of 3 concentrations of 10 different antibiotics the lower concentration showed higher rate of resistance in

FIGURE - VI


Pseudomonas aeruginosa
100
80
60
40
20
0
80
14.77
5.3
78.7
16
5.9
78.7
18
3.3
Lower concentration
Middle concentration
Higher concentration
Higher concentration
Middle concentration
Lower concentration

Pseudomonas, Klebsiella and *E. coli* strains though not much differentiation in sensitivity was noted between middle and higher concentration. (Chandrika 1984) (Figure VI)

Also, it is known that *P. anguillisceptica* is sensitive to chloremphenicol, tetracycline, dihydrostreptomycin, Kanamycin, colistin, furazolidone and moderately sensitive to Norobiocin and leucomycin and resistant to Penicillin, erythromycin, oleandomycin and sulphamethizole.

No relationship has been demonstrated between the predominant species and sensitivity or resistance towards antibiotics, as might be expected (Alexander, 1984). Indeed, the predominant bacteria are generally quite resistant to antibiotics.

Antagonistic effects of pathogenic Pseudomonas isolated was tested against test pathogens like *Vibrio anguillarum* H^{10}, *Cytophaga psychrophila, Edwardsiella tarda* and *Mycobacterium tuberculosis* (Table XV). Four cultures, out of the 13 tested, gave best results for *Vibrio anguillarum*, a potential pathogen causing Vibriosis in culture systems. Seven cultures inhibited *Cytophaga* and 6 isolates were found to inhibit *Edwardsiella* (Plates XIIa, XIIb, XIIIa, XIIIb)

Well plate method was found to be the more suitable method to identify the antagonistic activity of

microorganisms when compared to cross streaks assay method, as well plate method gave best results in the present study.

Shewan (1944) reported the role of Pseudomonas in Teleost spoilage. In the initial period, of spoilage (6-14 days) *Pseudomonas* and other bacteria were not reported. But in the later stage of 6-14 days, *Pseudomonas* occurred in the concentration of 6% of total and *Pseudomonas* dominated in the teleost spoilage by causing rapid proteolysis during 14-21 days. The increase in ammonia, due to deamination increased the number of *Pseudomonas* spp. and it formed 100% during the period of 14-21 days. *Pseudomonas* was the only genus to give free ammonia during the process.

The predominance of *Pseudomonas* in any environment is due to its antagonistic activity against other bacterial flora. The activity was exhibited well even towards a potential human pathogen, *Mycobacterium tuberculosis*, by six of the strains isolated in the present study.

Bactericidal action of a bacteriocin from Pseudomonas sp R^{10} produced siderophores and showed antimicrobial activity against *Aspergillus niger*. Friedman *et al.* (1989) showed that *P. aerugenosa, P. putida* and *P. fluorescens* showed antagonism towards food borne bacteria like *Salmonella* and *Staphylococcus*. Padilla *et al.*, (1996) showed that *Pseudomonas* species produced bacteriocin against several enteropathogenic bacteria. In the present study, out of 11 cultures studied, 4 cultures showed antagonism

against *Vibrio anguillarum*, 7 against *cytophaga*, 6 against *Edwardsiella* and *Mycobacterium* indicating the potential of *Pseudomonas* species in producing antagonistic compounds against other microbes.

Anti *Saprolegnia* activity of *P. fluorescens* was first identified by Hatai and Willougby (1988); these bacteria were considered relatively non-pathogenic to fish. In summary it appears that P. fluorescens is a natural competitor of *Saprolegnia* and in future it may be utilised for the biological control of pathogens in aquaculture.

Takahira Kunira et al., extracted antiviral agent 46 NW - 04 A from Pseudomonas fluorescens, but it was found that it had no antiviral activity against IHHNV.

The most commonly encountered bacteria in the environment, synthesising antibiotics are the species of Bacillus (60%), strains of *Pseudomonas* that liberate pyocyanin and related compounds (Alexander, 1984), which was found true in the present study. There is no evidence that the ability to produce antibiotics favours survival of the active cultures. Toxin synthesizers, despite their apparent competitive advantage are not particularly more common than the innocuous organisms. Antagonistic compounds even if produced, can be inactivated through adsorption, chemical reaction or by biological decomposition. Thus, a balance is maintained in the environment to protect the biodiversity of micro-organisms which stablise the eco-systems.

SUMMARY

1. The present study on ecophysiology of pathogenic Pseudomonas in seasonal and perennial ponds in Cochin was carried out in two selected ponds - perennial and Pokkali fields in Edavanakkadu and Manjanakkadu near Narakkal ($9^{o}55'$ to $10^{o}10'$N and $76^{o}10'$ to 76^{o} $20'$E) during May and June, 1997. Much emphasis was given to occurrence and distribution of pathogenic *Pseudomonas*. An attempt was also made to know the biochemical activity, antibiotic resistance and antagonistic activity. Data on physicochemical parameters were collected to find out the relationship if any, with pathogenic *Pseudomonas*.

2. Two selective media were used to retrieve pathogenic *Pseudomonas*. Pseudomonas agar + Triphenyl Tetrazolium chloride (TTC) and cetrimide agar + TTC. Pseudomonas agar and seawater agar gave best results for isolation of pathogenic *Pseudomonas* (Plate No.VII)

3. Pseudomonas agar and TTC media was found to be suitable for isolation of filamentous fungi also from sediments (Plates VIIIa, VIIIb, VIIIc, VIIId and VIIIe)

4. The count in perennial pond water ranged between 9 x 10^3/ml in early May to 140 x 10^3/ml in late June in SWA medium without TTC and with TTC supplemented SWA

medium respectively. It ranged from 60 x 10^3/ml to 300x10^3/ml, in the case of SWA supplemented with TTC. The count in Pseudomonas agar was very low in the final observation (36 x 10^3/ml); when Pseudomonas agar was supplemented with TTC, the count was more, when compared to ordinary Pseudomonas agar, and it ranged from 14 x 10^3/ml to 120 x 10^3/ml. (Figure II)

In perennial pond sediment, with seawater agar, the count was very low throughout the observation except in late June, when the count recorded was as high as 300 x 10^4/gram. When SWA was supplemented with TTC, the counts recorded were very low except in late June, the count being 75x10^4/g. (Figure III). Pseudomonas agar also registered comparatively lower counts.

In Pokkali pond water, the counts ranged from 43x10^3/ml to 165x10^3 ml. with SWA. In PA supplemented with TTC high counts (150x10^3 ml) were obtained in early May (Figure IV).

In Pokkali sediment, generally count was very low in all the four observation. Seawater agar supplemented with TTC gave the best results with a highest count of 140 x 10^4/gr. When Pseudomonas agar was used highest count obtained was 120 x 10^4/g. and when supplemented with TTC, counts upto 125 x 10^4/gram were obtained in early June. (Figure IV)

5. Maximum number of isolates occurred in surface water, when compared to sediment. The sheer dominance of *Pseudomonas* in water have led to an over emphasis of its ecological significance.

6. Totally 31 isolates were retrieved from the three observations out of which 11 isolates were identified as pathogenic Pseudomonas. *Pseudomonas fluorescence* and *Pseudomonas putida* were predominant (38.46%) in the present study. *P. anguillisceptica, A. piscida* and *A. faecalis* formed 7.6% each. (Table XIII)

7. Out of the 11 pathogenic Pseudomonas isolated, three exhibited greenish yellow flourescent pigment (Plate IX and X) and dipotassium hydrogen phosphate was found to favour enhancement of fluorescent production. Isolates were typed as *Pseudomonas fluorescens* with confidence since they fluoresced under ultra-violet light.

8. Out of the seven nitrogenous substances tested to find out metabolic activity, five were organic compounds and two inorganic salts. Inorganic salts were degraded quickly, when compared to organic compounds. (Table VII)

10. Sodium chloride tolerance test was carried out for a species level identification (Table IX). It was found that most of the isolates were able to tolerate

sodium chloride upto 5% level. (69.23%), and only one showed affluent growth at 5% concentration. Not much growth was noticed beyond 5% concentration of Nacl, only one isolate showed good growth at 7% sodium chloride concentration. Growth was completely absent in 10% Nacl. It was also noted that, most of the pathogenic *Pseudomonas* exhibited good growth when sodium chloride was omitted in the medium.

9. Elevated temperature test showed that at $37^{o}C$, 38.46% exhibited moderate growth but at $5^{o}C$ not much growth was there. Poor growth was exhibited by 38.46% of the isolates at $37^{o}C$ (Table VIII). So all pathogenic *Pseudomonas* can be classified as mesophilic in nature.

11. Proteolytic activity was more compared to amylolytic activity (Plate No. XI) (Table No. XI)

12. From the antibiotic sensitivity test it was found that 100% of the isolates were **resistant** to Penicillin and Ampicillin. But Kanamycin was resisted by 46.15% of the isolates. (Table XIV)

13. No relationship has been demonstrated between the predominant species in the sediment and their sensitivity or resistance to antibiotics. The predominant bacteria were quite **resistant** to antibiotics like Kanamycin or Ampicillin.

14. Antagonistic activity was tested using 4 test pathogens like *Vibrio anguillarum* H^{10}, *Cytophaga*, *Edwardsiella* and *Mycobacterium*. 38.76% were found to have inhibitory activity against *Vibrio anguillarum* H^{10} and 53.84% were having inhibitory activity against *Cytophaga*. 46.15% were having inhibitory activity against *Edwardsiella* and *Mycobacterium*. (Table XV)

15. There is no evidence that the ability to produce antibiotics favours the survival of active culture. Toxin synthesizer despite their comparative advantages were particularly not common than the innocuous organisms.

16. Dr. D.J.W. Moriarty (1997) is of view that the application of microbial bio-technology is favourable and necessary and the research field of microbial ecology will advance with the demands that the aquaculture industry is placing on it. In the present study a large group of organisms responsible for the majority of the recognised bacterial diseases like acute septicaemia causing high mortalities in fishes, brown-spot diseases in shrimps and bacillary necrosis in molluscs were studied with intensity charts and bio-chemical activity tables. Determination of oxidase activity, oxidative or fermentative (O-F) attack on glucose and motility categorised pathogenic *Pseudomonas* from these culture systems.

17. In summary it appears that *Pseudomonas* spp. is a natural competitor to control other pathogens, and in future it may be utilised for the biological control of pathogens in aquaculture.

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